Analysis of Myelin-Reactive T Lymphocyte Function in Models of Multiple Sclerosis

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

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Abstract

Immune tolerance to self antigens prevents the onset of autoimmune diseases such as Multiple Sclerosis (MS). There are three branches of tolerance which allow the auto-aggressive potential of T lymphocytes to be limited; these are death, anergy-adaptation and regulation.

The main body of this work attempts to clarify a role for adaptation in maintaining the sensitivity of the autoreactive T cell repertoire below a 'threshold for harm' in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE). The well defined myelin basic protein (MBP) Ac1-9 epitope altered peptide ligand (APL) system has been used to develop a model allowing the examination of mechanisms underlying the adaptation of cells. Previous data showed immunisation with the 4Lys (wild-type) epitope mediated disease whereas a superagonist APL with a tyrosine substitution at position 4 (4Tyr) did not, despite showing potency *in vitro*. This was shown to be a result of both activation induced cell death and adaptation. Here an *in vitro* model was developed using MBP-reactive TCR transgenic cells to make predictions about the mechanisms underlying adaptation. These data lead to the conclusion that T cells can adapt (become less sensitive) either before or after encounter with the wild-type peptide, leading to a reversal of their pathogenic potential.

The MBP APL system and MBP reactive transgenic cells were also used to assess the contribution of epitope spreading in a relapsing-remitting (RR) model of EAE induced with proteolipid protein. The cells were tracked and changes in phenotype and behaviour were monitored. The data show that disease induced with one antigen can be manipulated with cells relevant to a different antigen and that bystander suppression may be an effective weapon in controlling the progression to RR-EAE.

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ABBREVIATIONS USED

ACAD	activated-cell autonomous death
AICD	activation induced cell death
APC	antigen presenting cell
APL	altered peptide ligand
BBB	blood brain barrier
CFA	complete Freund's adjuvant
CNS	central nervous system
CTLA-4	cytotoxic T lymphocyte antigen-4
EAE	experimental autoimmune encephalomyelitis
DC	dendritic cell
FACS	fluorescence activated cell sorting
IFA	incomplete freund's adjuvant
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
LN	lymph node
MACS	magnetic activated cell sorting
MBP	myelin basic protein
MFI	mean fluorescence intensity
MHC	major histocompatability complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PBS	phosphate buffered saline
PLP	proteolipid protein
PTx	pertussis toxin
SEM	standard error mean

SD	standard deviation
TCR	T cell receptor
TGF-β	transforming growth factor β
Th	T helper cell
TLR	toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TNF	tumour necrosis factor
Treg	regulatory T cell
Tr1	type 1 regulatory cell

Chapter 1:

Introduction

1. Introduction

The immune system consists of a highly synchronised system of layered defences whose combined purpose is to identify and eliminate foreign elements such as bacteria and virions from the host and to eradicate malfunctioning host cells.

Physical barriers provide the first layer of defence; skin prevents the majority of infections, whilst sneezing and coughing can eject others. Many bodily secretions also carry antimicrobial agents, tears carry lysozymes and skin secretes β-defensins.

The second layer is the innate immune system. This system serves to recruit cells to the site of infection through the release of chemical signals; it also activates the complement cascade. Within this system certain white blood cells, phagocytes, patrol the host to detect foreign matter, then eat and digest this matter. Other cells, dendritic cells (DC), are able to pick up foreign antigen and in response can release signalling molecules into the bloodstream. This brings us to another role of the innate system; the activation of adaptive immune responses.

The adaptive immune response is the third tier of the immune system; it consists of bone marrow derived B and T lymphocytes and is activated by cells of the innate response which present foreign antigen to T lymphocytes. The presentation of antigen activates the T lymphocytes and allows them to produce cytokine signals which enhance the defensive properties of other cells. B lymphocytes detect antigen via antibodies on their cell surface and can process these to present to T lymphocytes in order to aid their own activation.

1.1. Multiple Sclerosis

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) in the developed world. It affects approximately one in 1000 people in Europe, North America and Australasia, a total of around 2.5 million people worldwide [1, 2]. It is unknown whether MS is a result of environmental [3] or genetic factors (35% concordance in monozygotic twins [4]) , infectious agents [5] or a combination of all of these. The disease is characterised by 'acute focal demyelination and axonal loss culminating in chronic multifocal sclerotic plaques'[2] and perivascular inflammation. The plaques locate to optic nerves, brainstem, cerebellum and spinal cord white matter, the location explaining clinical outcomes such as visual impairment and paralysis due to the loss of signal integrity between nerve cells in the CNS.

MS is generally divided into two categories, relapsing-remitting (RR) and chronic-progressive (CP). 80% of patients have the RR MS form which usually affects young adults in whom symptoms flare up for several days and the disease can go into remission for many years. With CP MS in all cases the symptoms continue to worsen without remission, however as the severity and timing is variable it is divided into a further 3 sub-categories; primary-progressive (PP), secondary-progressive (SP) and progressive relapsing (PR) [2, 6].

A single disease or various diseases with similar symptoms?

The clinical symptoms of MS vary between patients and recent literature indicates that the various forms of MS may in fact constitute different diseases. This suggestion is supported by distinct categorisation of disease lesions into 4 pattern classes. Pattern 1 is macrophage mediated; 2 is both macrophage and antibody mediated and constitutes the highest number of cases; 3 shows distal oligodendropathology and apoptosis; 4 shows primary oligodendroglia degeneration

[7]. Patterns 1 and 2 are associated with T cell mediated encephalomyelitis whereas 3 and 4 are associated with viral damage. Emerging candidates as an alternative diagnosis to MS appear to be Neuromyelitis Optica (NMO) and Balo's Concentric Sclerosis (BCS). BCS lesions are typically pattern III lesions associated with increased inducible nitric oxide synthase leading to mitochondrial dysfunction and hypoxia like tissue injury [8]. NMO lesions show 'rim' and 'rosette' pattern II lesions in both the grey and white matter of the CNS. NMO can also be distinguished from MS by the presence of NMO-IgG which is absent in MS cerebrospinal fluid (CSF). This NMO-IgG targets the aquaporin-4 water channel (AQP4). AQP4 is manly found in the periventricular and periaqueductal areas of the brain and spinal cord and is concentrated at the astrocytic foot processes which line the outside of the blood brain barrier [9].

Genetic Associations within MS

The involvement of genetic factors in MS has long been proposed [10] and has been supported by the predominance of the disease in women over men [11] .

The Human leukocyte antigen (HLA)-DR2 in chromosome 6 is represented at increased frequencies in MS and is therefore suggested to confer susceptibility to MS. The DR3 molecule has also been shown to be associated with CP MS [12]. Increasingly the question in MS appears not to be which genes are responsible for the disease but which genes are responsible for the various aspects of the disease. The strong linkage disequilibrium within the HLA has made pinpointing exact genes difficult.

The first indication of a role for HLA-DR in the progression of MS came from transgenic mice bearing HLA-DRA-IEα and HLA-DRB1*0401-IEβ chimeric genes [13].

To enable definition of gene function, Fugger and colleagues later developed a set of transgenic mice which feature HLA-DR2 elements (DRB1*1501 and DRB5*0101) and T cell receptor (TCR) (Hy2E11 TCR) from MS patients. These humanised mice contain either one or both of the HLA alleles. DRB1*1501 mice show clinical signs of severe disease whereas DRB5*0101 mice do not develop disease. The addition of the *0101 to the *1501 reduces the severity and the incidence of disease. This suggests that in humans where both elements are present the *1501 is the causal gene and that *0101 acts as a genetic modifier. The *0101 causes apoptosis in disease relevant T cells and therefore can be considered an implement of peripheral tolerance [14].

Other specific risk alleles were identified in a DNA micro-array analysis of 931 family trios. This study confirmed that 57% of those screened had the HLA-DRB1*1501 allele. Other alleles identified as having a significant association with MS encoded the IL-2R α chain (CD25) and the IL-7Rα chain. Any impact on IL-2Rα may have an effect on $CD4^{+}CD25^{+}$ Regulatory T cells and therefore on peripheral tolerance. IL-7 has an effect on homeostasis of the T cell memory pool and potentially a role in generating autoreactive T cells [15].

Current therapies for MS

Many potential treatments have been applied to MS, however there is no cure currently available and the two best known licensed treatments are Beta Interferon and Copaxone. Trials with Campath-1H are currently taking place.

The beta interferons (Interferon β1a and β1b) were the first drugs approved for use in cases of RR MS and are known to reduce the destructive activity of IFN-γ. Beta interferon reduces relapse rates as well as slowing the accumulation of disability. However recent reports have indicated that due to the action of anti-beta interferon antibodies the efficacy of this drug is reduced in some patients [16].

Glatiramer acetate (Copaxone) is a synthetic amino acid co-polymer which is made up of L-alanine, L-lysine, L-glutamic acid and L-tyrosine in a similar molar ratio as they would be found in MBP. It has similar effects to beta interferon. It is suggested that Copaxone exerts its effects by inducing a T helper (Th) 2 cell response to combat the Th_1 response which mediates disease [17]. Some studies suggest that it may also competitively bind MHC molecules thereby preventing self antigens from binding [18].

Campath-1H is a rat monoclonal antibody which was humanised and developed to treat human malignancies by lysing T lymphocytes [19]. It targets the CD52 antigen (integral membrane protein used in signal transduction) on lymphocytes and mediates sustained T cell depletion through either complement or cell mediated cytotoxicity. Campath-1H is much more powerful than the other drugs available because the CD52 antigen is expressed on a wide range of immune system cells including all lymphocytes, DC and macrophage.

Rituximab (IDEC-C2B8) is known to target the CD20 transmembrane phosphoprotein that is expressed only by pre-B and mature B cells. This genetically engineered chimeric murine / human IgG_1 kappa monoclonal antibody binds complement and therefore can mediate B cell lysis [20]. Originally this drug was developed as an alternative to chemotherapy treatment non-Hodgkin's Lymphoma and was the first licensed monoclonal antibody used to treat cancers [21]. Clinical results from studies using Rituximab have shown significant decreases in B cell as well as T cell (55% decrease) levels. The B cells were eliminated for up to 9 months after therapy (not memory B cells however as they express no CD20). A disadvantage of the use of this drug is that it may not be effective in combination therapy, its use alongside beta-interferon showed the emergence of anti-beta-interferon antibodies despite the apparent depletion of B cells.

Natalizumab is another more recent addition to the drug arsenal against MS. It is a monoclonal antibody directed against the adhesion molecule VLA-4. VLA-4 expression is required by T cells to migrate from the peripheral lymph nodes, across

the blood brain barrier and into the CNS. The use of natalizumab alone showed dramatic reductions in relapse rates per annum, this reduction was similar to that seen when natalizumab was used in conjunction with beta-interferon implying that combination therapy was no more effective than natalizumab alone. However in two cases (from 1000) severe progressive multifocal leucoencephalopathy was seen due a JC virus infection [22].

Fingolimod (FTY720) is an immunomodulatory drug which down-regulates the expression of sphingosine 1-phosphate receptor. This has the effect of sequestering mature T lymphocytes within the secondary lymphoid tissues and thymus thereby preventing their migration into the CNS. This is confirmed by histological data which shows a reduction in the number of plaques within sick mice treated with FTY720 compared to those that are not treated. The plaque being a result of and therefore an indicator of T cell infiltration into the CNS [23].

1.2. Experimental Autoimmune Encephalomyelitis (EAE)

EAE is the animal model of choice to study MS. EAE has its origins in the observations at the turn of the 20th Century that a small number of people, who had been vaccinated against rabies, were showing signs of paralysis. In 1905, Remlinger [24] postulated the disease to be a result of CNS tissue contaminating the vaccine. In 1928, Stuart and Krikorian reported that when rabbits were repeatedly inoculated with human spinal cord or sheep's brains that the treatment occasionally resulted in paralysis [25].

Later, spurred by his own recurring incidences of muscular atrophy [26], Rivers showed that demyelinating disease was inducible in primates using injections of rabbit CNS [27]. Rivers used as many as 50-80 injections of CNS tissue before he observed a result. Later studies showed that a single administration could be used if accompanied by complete Freund's adjuvant (CFA) [28]. With advances in the

understanding of the myelin sheath we are now able to induce EAE with specific myelin antigens in conjunction with CFA and Pertussis Toxin (PTx); this is known as the active induction of EAE.

Lymphocytes have been known to play a central role in the dissemination of MS and EAE model since Paterson's experiments in the 1960s which showed that lymph node cells could transfer disease between rats [6, 29, 30]. Paterson's work was also the first description of the passive induction of EAE however this programme of work did not clarify the group of lymphocytes responsible for the manifestation of disease.

T cell dependency of disease was shown in experiments using thymectomised irradiated rats reconstituted with either normal lymphoid cells or those depleted of T cells (by treatment with anti-rat thymocyte serum) from mice previously challenged with MBP and CFA. Non-manipulated cells potentiated normal EAE and antibody production whereas depletion of T cells resulted in the prevention of EAE and the ablation of antibody production [31] .Later work blocking T cell surface molecules, such as CD28 and CD40L, with antibodies and the depletion of $CD4^+$ post induction of EAE has also shown the central role of firstly T cells and then $CD4^+$ cells within the progression of the disease [32-34].

Today passive transfer EAE experiments are conducted by the adoptive transfer, into naïve mice, of T cells isolated from myelin antigen primed mice. The CFA used in the active model of EAE contains *Mycobacterium tuberculosis* which aids the initial development of an immune response upon administration with the relevant myelin antigen. The passive model is advantageous because it bypasses the need for the introduction of non-disease relevant antigen.

Although the EAE disease model has been replicated in many animals, the pattern of disease progression and the histopathology varies significantly between each species. All models share a destruction of the myelin sheath, lesions in the CNS and the presence of immunoglobulin in the CNS and cerebrospinal fluid [35].

EAE shares many of the characteristics of MS, including the widespread foci of inflammation and demyelination accompanied by perivascular infiltration of mononuclear leukocytes. Disease course is often monophasic but can also have relapsing and chronic phases depending on the antigens and species used to induce disease.

It must be noted that EAE is not MS nor is it a perfect model of MS. However the various models of EAE are useful in representing various aspects of MS.

1.2.1. Identification of Myelin Autoantigens

Three major autoantigens involved in MS and EAE have been identified; Myelin basic protein (MPB) [36], proteolipid protein (PLP) [37] and myelin oligodendrocyte glycoprotein (MOG) [38]. These CNS antigens have been relatively hard to isolate because of the high lipid content of the myelin sheath.

MBP, discovered in 1962 by Einstein *et al*, is a major protein constituent of the myelin sheath (around 30%) and is expressed both in the central and peripheral nerves. EAE can be effectively induced with MBP and adjuvant in a wide range of animal models [30, 36, 39]; most work in EAE has been done on MBP due to its ease of isolation.

PLP makes up 50% of the myelin sheath protein and because of its hydrophobicity interacts with myelin lipids and is also difficult to purify. This hydrophobic nature of this integral membrane protein means that it is difficult to work with due to insolubility [40-42].

MOG composes less than 0.05% of the myelin sheath protein, however it induces EAE in many strains and species of animals [43]. It has been suggested that the severity of the disease caused by MOG may be due to the accessibility of the protein

to antibodies, therefore MOG induced disease may be antibody and complement mediated [40].

The phenotype of disease depends on the peptides used to induce EAE; the lesions in MBP and PLP induced disease are seen primarily in the spinal cord whereas those from MOG induced disease are seen in optic nerves as well as in the spinal cord [43]. As discussed later, T cells recognise short peptide sequences (9-11 amino acids), generated by antigen processing, displayed on MHC class II molecules. Epitope mapping studies have allowed the identification of distinct T cell epitopes that are able to mediate disease.

The first myelin protein in which an encephalitogenic region was defined was MBP [44] in experiments using synthetic peptides to induce EAE in guinea pigs. Evavold & Allen used analogue peptides with amino acid substitutions within this model to demonstrate the requirement for single residues in disease progression and thereby essentially created the first altered peptide ligands (APLs) [45]. It was more than a decade later that Zamvil and colleagues were able to identify the most commonly used murine encephalitogenic region of MBP, the n-terminus Ac1-9 epitope on H-2^u MHC genetic backgrounds [36].

Later studies in H-2^s (SJL) mice identified the encephalitogenic $PLP_{139-151}$ sequence [41] as well as the MOG_{92-106} [46]. The identification of the MOG_{35-55} sequence allowed for the expansion of EAE studies into the C57BL/6 murine strains [47]. This has allowed the expansion of studies into EAE using well developed gene knockout and transgenic variants of the C57BL/6 mouse.

The identification of sequences with the potential to help induce autoimmune pathogenicity enables the induction of EAE with synthetic T cell epitopes, CFA and PTx. Synthetic peptides have also helped to overcome the problem of isolating infrequent and often hydrophobic proteins from the myelin sheath.

1.2.2. TCR Transgenic Models of EAE

The use of TCR transgenic mice has helped to overcome the problem of limited numbers of disease relevant cells in many models including EAE [48]. Paradoxically, in some transgenic mice, the large number of these antigen-relevant cells leads to poor T cell activation, upon challenge, due to competition between the TCRs to bind to MHC.

The Tg3 and Tg4 models constructed by Liu *et al* [49] have an encephalitogenic TCR, on the B10.PL (I-A^u)background, which recognises the encephalitogenic MBP_{Ac1-9} peptide. Created from a MBP_{Ac1-9} reactive T cell clone, all the Tg4 $CD4^+$ cells were found to have the Vβ 8.2 chain [50]. These mice require immunisation with peptide before EAE could be induced.

The transgenic mouse relevant to MOG_{35-55} is the 2D2, made by Betelli *et al*. The TCR in this model consist of a Vα3.2 and Vβ 11 on the I-A^b (C57BL/6) background [43]. However studies in our group have suggested that these mice have cells deficient in being able to cross the blood brain barrier and are deficient in inducing effective disease in some murine strains of the same background (unpublished findings).

A transgenic mouse for PLP was created using T cell clones, derived from SJL mice, which expressed TCR carrying Vα4 and Vβ6. They respond to $PLP_{139-151}$ on the I-A^s background. These mice developed spontaneous EAE with too great a frequency to be maintained on the SJL background [51].

Fugger's humanised model of EAE has already been discussed earlier in this chapter (Section 1.1).

1.3. Theiler's Murine Encephalomyelitis Virus (TMEV)

TMEV is an endogenous mouse pathogen which targets antigen presenting cells (APC), in particular quiescent microglia, in order to establish a persistent, life-long, infection in the CNS APCs; this infection results in up-regulation of the innate immune response accompanied by the production of cytokines to activate T cells and cell surface receptors facilitating antigen presentation to T cells [52].

In SJL mice, infection with the wild-type TMEV disease develops between 4 – 5 weeks post-infection. TMEV relevant Th_1 cells target the virus in the CNS and produce pro-inflammatory cytokines which activate macrophages. These activated macrophages cause bystander damage to the CNS resulting in a breakdown in the myelin sheath and immune responses are directed to myelin epitopes via epitope spreading. T cell proliferative responses to the viral epitope are seen within 14 days of infection and last through to at least day 90. Responses to PLP epitopes are seen around day 50 post-infection and subsequently spread to other myelin epitopes as myelin sheath damage is increased.

Miller *et al* developed a molecular mimicry model of MS by engineering a non-pathogenic variant strain of TMEV encoding a 30-mer peptide incorporating the $PLP_{139-151}$ epitope. Infection with this PLP-TMEV reduces the onset of disease to a week; autoreactive Th_1 cells are rapidly directed to the CNS and initial myelin damage is followed by epitope spreading resulting in a relapsing-remitting disease [52, 53]. Building on this and on earlier work by Carrizosa *et al* [54], Miller's group engineered another variant TMEV to further illustrate the potential of molecular mimicry in disease induction. These experiments used TMEV encoding a *Haemophilus influenzae* mimic peptide ($HI_{574-586}$) which shared only 6 of the 13 amino acid residues of $PLP_{139-151}$ [53] including the primary TCR and MHC contact residues.

Mice infected with this virus exhibited early onset of disease and a cross-reactive CD4+ T cell response to $PLP_{139-151}$. When emulsified with CFA, $HI_{574-586}$ fails to

induce demyelination in the CNS of SJL mice even when administered several times. The fact that the combination of TMEV and $HI_{574-586}$ can cause disease highlights the role of a 'natural' infection in the induction of autoimmune disease via molecular mimicry and possibly within epitope spreading [53, 55].

1.4. $CD4^+$ T cell Activation

T lymphocytes act to direct the adaptive immune response by working as antigen receptor bearing cells in synchronisation with APC. This relationship is essential for the clonal expansion of T lymphocytes and progression into effector T cells [56]. In order to facilitate this interaction the immune system is required to arrange an encounter between one of 1 in 100,000 (approximately) peptide-relevant T cells and an APC presenting the relevant peptide in association with MHC molecules [57].

Naïve T cells mainly recirculate through the blood and the secondary lymphoid tissues. The expression of chemokine receptors (CCR7) and adhesion molecules (CD62L) directs the circulation between the secondary lymphoid tissues through migration across the high endothelial venules (HEV). On entry to the LNs, T cells are attracted by chemokines (e.g. SLC/CCL21, the ligand for CCR7) to the T cell areas where they undergo multiple short encounters with DC and scan for antigen. If no antigen (foreign or domestic) is present the T cells remain in the nodes for up to 20 hours and then re-circulate [58].

Upon antigen presentation CD44 (adhesion molecule) and CD69 expression is up-regulated and T cells slow down and have been observed to make longer contacts with DC. T cells also produce IL-2 and CD25 (the IL-2 Receptor α-chain) although no proliferation takes place [59]. With the up-regulation of CD25 the cells become more responsive to IL-2. This phase lasts between 8 and 24 hours. At 24 hours rapid proliferation of antigen-relevant T cells is seen and although the progeny express less CD25, CD69 and CCR7; CD44 remains high [60]. The activated cells start to express other chemokine receptors which enable migration of the cells to the lymph node follicles (CXCR5) or to peripheral sites of inflammation (CCR7, CCR5 and CXCR3) [60].

1.4.1. Requirements for T cell Activation

Signal 1 and Signal 2

To enable the activation of $CD4^+$ T lymphocytes, two signals are required; Signal 1 is provided through the interaction between the T cell receptor complex and peptide presented within the MHC Class II groove on the surface of an APC.

Signal 2 also provided by the APC is mediated by the cross-linking of CD28, leading to T cell activation and proliferation [61]. Further work identified B7 molecules (CD80 and CD86) as ligands for CD28 [62]. The CD28 signal is required for the production of IL-2 that drives T cell proliferation [63, 64]. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is known to act with CD28; CTLA-4 has a greater affinity for B7 and through competitive binding it induces an inhibitory effect on signalling.

Von Boehmer postulates three theories as to the function of CTLA-4 based on its interaction with the B7 molecules: (1) naturally occurring regulatory T cells (nTregs) may interact with effector cells at an APC and then either act directly with the effector cell or through the APC. (2) Suppressor cells may not be present at all and effector cells may suppress each other through CTLA-4 and B7 interaction. (3) In APC mediated suppression, the suppression of effector T cells would appear to be the result of the up-regulation of indoleamine 2,3-dioxygenase (IDO) which metabolises tryptophan, an amino acid required for T cell activation [65].

Further research has shown that co-stimulation is far more complex than simply the interactions between CD28 and B7; there are many other costimulatory or accessory molecules involved. There are now suggested to be two main groups of costimulatory molecules; the immunoglobulin superfamily (including CD28 and CD2) and the tumour necrosis factor-R superfamily (OX40, CD137 and CD27) [66]. This latter group are all shown to boost the production of cytokines and proliferation of reactive T cells when cross-linked. For example, OX40 deficient T cells have

reduced proliferation and undergo apoptotic cell death at days 4-5 after activation because OX40 controls the anti-apoptotic BCL-2 family of molecules [67] [68] .

Adhesion molecules, such as CD58 (CD2 ligand) [69] and ICAM-1 (LFA-1 ligand)[70] are also known to be able to contribute to Signal 2 and in turn allow the formation of the immune synapse. The need for prolonged cell contact through cell adhesion molecules is magnified by the slow binding kinetics of the TCR-peptide-MHC interaction [69]. The presence of Signal 1 alone results in T cell anergy or death *in vitro* and tolerance *in vivo* [71].

Signal 3 and the CD4$^+$ Subsets

Signal 3 is a reference to signals that are transmitted from the APC to the T cells which determine the naïve T cells path of differentiation into three main effector T cell groups, Th_1 cells, Th_2 cells or Th_{17} cells. Signal 3 is transmitted through the production of cytokines by the APC; the cytokine milieu that is created dictates which subset the T cells belong to and which combination of cytokines the T cell itself will release upon activation (Figure 1.1).

IL-12 is an example of a cytokine, released by DC, which is able to deliver signal 3 and promote Th_1 cell expansion. The Th_1 subset is characterised by the production of tumour necrosis factor alpha (TNF-α) and IFN-γ. IL-12 augments the production of IFN-γ which up-regulates MHC Class II and co-stimulatory molecule expression by microglia and astrocytes [72] and thereby increases the presentation of myelin antigens to cytotoxic T cells. IL-12 also promotes the expression of the IL-18 receptor which again aids to promote the production of IFN-γ. IL-4 promotes the Th_2 phenotype characterised by production of IL-4, IL-10 and IL-13. [6, 73-76].

Despite EAE appearing to be the prototypic Th_1-driven disease, IFN-γ deficient mice have been shown to be susceptible to a more progressive form of EAE with large inflammatory infiltrates in the CNS [77]. Initially these findings were supposed to be a result of the regulatory functions of IFN-γ [78] or its ability to induce apoptosis in

reactive T cells[79]. In addition, the injection of IFN-γ during chronic inflammation is known to block EAE [80]. The answer to why IFN-γ deficient mice suffer from EAE may be identified by the recent elucidation of the Th_{17} subset of $CD4^+$ lymphocytes.

In 2003, Aggarwal *et al* [81] showed that IL-17 production was enhanced from memory $CD4^+$ cells but not from naïve $CD4^+$ cells by the actions of IL-23. IL-23 was shown to be composed of the p19 and p40 subunits, the latter which it shares with IL-12. The p35 subunit of IL-12 is not shared however and this explains the findings that IL-12p35 deficient mice were previously observed to be susceptible to EAE [82] whereas p40 deficient mice were resistant to disease induction [83].

IL-23 is secreted by activated macrophages and DC [84] and Langrish *et al* [85, 86] suggest IL-23 acts on previously activated cells (before their final differentiation to Th_1 phenotype) to generate a Th_{17} subset. The suggestion of this subset had already been made in 2000, when it was observed that naïve T cells primed with *B. burgdorfei* lysates produced larger amounts of IL-17A than T cells primed under Th_1 and Th_2 conditions [87]. This model would predict that T-bet expressing (pre-Th_1) cells would express both IL-12R and IL-23R on their surfaces and depending on the cytokine milieu would differentiate into Th_1 or Th_{17} cells.

Distinct roles for both Th_1 and Th_{17} subsets are suggested by the factors they express; IL-23 polarised cells express high amounts of IL-17A and IL-17F as well as TNF-α. IL-12 polarisation of cells promoted the expression of IFN-γ, granzyme F, granzyme G, TNF-related apoptosis inducing ligand (TRAIL) and FAS ligand. A role for IL-17 was confirmed when EAE was induced by the passive transfer of IL-17 producing $CD4^+$ cells [85].

A key function of T cells in EAE appears to be the activation of macrophage. These cells are activated by IFN-γ; they in turn produce pro-inflammatory mediators including IL-1, IL-6, Nitric Oxide (NO) and TNF-α [88] as well as being involved in complement and antibody induced phagocytosis [89].

TNF-α has been shown to both mediate and control levels of inflammation [12]. It has cytotoxic effects on oligodendrocytes in the CNS that leads to apoptosis [90]; it also promotes the production of NO from macrophage that mediates further damage to these cells and causes demyelination [91]. Human studies show that the administration of anti-TNF-α antibodies results in immune activation. However in some animal models, these antibodies have been shown to ameliorate EAE possibly due to auto aggressive T cell apoptosis induction [92]. IL-1β [93] and IL-6 [94] are both shown to induce glutamate. Glutamate is a neurotransmitter which in excess causes neuronal cell death [88] and hence damage in the CNS.

IL-10 inhibits Th_1 cell proliferation as well as limiting Th_1 cytokine production. It also act on macrophages to prevent the production of pro-inflammatory cytokines, down-regulate MHC class II and co-stimulatory molecules [95]. Perhaps more importantly, the presence of IL-10 during T cell activation results in anergy or non-responsiveness in treated cells [96]. IL-10 deficient mice have been shown to develop a more severe form of EAE than wild-type mice and the T cells from these mice undergo greater antigen-specific proliferation and cytokine production when stimulated *ex vivo* [97]. IL-10 can be produced by T_R1 cells [98] or by B cells. The Anderton group have used B cell chimeric mice, in which the B cell compartment was IL-10 deficient, to show that disease recovery is impaired despite the presence of IL-10 from other sources [99].

Another cytokine implicated in the remission stage of EAE is transforming growth factor β1 (TGF-β1). The administration of TGF-β1 blocking antibodies increases the severity of disease whereas the administration of TGF-β1 ameliorates disease [100].

The signals received by the TCR, co-stimulatory molecules and cytokine receptors are collated to initiate the differentiation of naïve T cells into the various subsets. Th_1 polarisation is a result of the action of IFN-γ on signal transducer and activator of transcription 1 (STAT1) [101]. This, together with TCR signalling events, results in the up-regulation of the T-box transcription factor T-bet in naïve T cells [102]. T-bet

induces IFN-γ and has also been shown to induce chromatin remodelling of the gene encoding IFN-γ [103].

The actions of IL-4 on STAT6 result in the polarisation of naïve T cells to the Th_2 phenotype [104]. This results in the selective expression of c-MAF, a member of the basic-region leucine-zipper family [105] and GATA3 [106]. The former is known to transactivate the IL-4 promoter [105] whereas the latter transactivates the IL-5 promoter [107].

The recently discovered Th_{17} lineage is dependent on STAT3 and STAT4. TGFβ1 is always required to signal through STAT3 but it retains the flexibility to work in association with either IL-6 from DC or IL-21 from NK cells to differentiate the naïve T cells into Th_{17} cells [108]. For the transduction of signals through STAT4, TGF-β must work with IL-23 [109]. The transcription factor RORγt has also been identified as being specifically expressed in Th_{17} cells and as a regulator of Th_{17} differentiation. Mice lacking RORγt were shown to have a less severe course of autoimmune disease and failed to produce Th_{17} cells which could infiltrate tissue [110].

Figure 1.1

1.4.2. Molecular Basis of T cell Recognition

The specificity of a T cell is determined by its clonotypic TCR. The TCR molecule consists of an α and a β polypeptide chain, each containing a variable (V) and constant domain. The V chain uses complementary determining regions (CDRs) to achieve variability between TCR; CDR1 and CDR2 through the germ-line and CDR3 through somatic mutation [111]. These variable regions on $CD4^+$ T cells recognise antigenic peptide bound within the groove of an MHC Class II molecule. The binding groove of the Class II heterodimer (α and β chains) consists of the floor (beta-pleated sheet) and the walls (α helices). The binding grove of the Class II molecule has open ends allowing the binding of peptides of variable length (10-15 amino acids) [112].

Several 'pockets' within the binding groove determine which peptides can bind. Peptides need only contain two or three critical amino acid residues (a motif) correlating with the pockets in order to bind to a given MHC class II molecule [113]. Allotypic variation in the peptide binding grooves of different class II molecules determines which peptide will bind which MHC molecule and is central to MHC-restriction in T cell reactivity. With the peptide bound between the MHC class II, the TCR positions itself so that the CDR1 and CDR2 are over the N- and C- termini of the peptide and interacting with the HLA α and β chains [114, 115]. The CDR3 chain interacts with the peptide to determine the specificity of the interaction [116]. In humans the MHC class II loci within HLA complex are DP, DR and DQ homologues in the mouse are the I-E and I-A H-2 gene loci respectively.

In nearly all autoimmune diseases susceptibility to disease is associated with certain HLA molecules [117].

$CD4^+$ T cells recognise peptides in the context of MHC class II. Although many cell types can express MHC class II under extraordinary conditions, the true APC are B cells, macrophages and DC, since their MHC class II expression is constitutive. In

addition to signal 1 APC must provide signal 2 (costimulation). The three types of APC vary in their ability to provide both signals 1 and 2.

These cells are able to uptake antigen via receptor mediated endocytosis, phagocytosis and macropinocytosis. Proteins must be processed to provide peptide fragments for MHC class II loading. Antigen processing is facilitated by a battery of proteases within the endocytotic pathway. MHC class II peptide loading occurs in low pH endosomal compartments [116] by removal of the CLIP peptide which protects the MHC class II binding cleft during biosynthesis. MHC class II is known to recycle from the plasma membrane allowing the opportunity for possible peptide exchange at, or close to, the cell surface.

B cells are inefficient at antigen uptake unless it is via their antigen-specific surface immunoglobulin (Ig) receptors. The binding of antigen to these receptors leads to internalisation of the receptor followed by rapid presentation of the peptide on MHC class II. This means that the B cells can uniquely focus on presenting specific antigens to elicit T cell help during the humoral response. B cells are also aided in their presentation capability by the high expression of MHC class II and costimulatory molecules upon activation [118].

B cells have the potential to influence the progression of EAE via 3 mechanisms; they can produce pathogenic autoantibodies; they can act as APC, as described above, to activate auto-aggressive T cells; or they can play a role in mediating recovery from disease (discussed later). The APC function of B cells has been the subject of much scrutiny; initial experiments in which mice were depleted of B cells, using anti-μ antibodies, suggested that B cells were required as both APC and producers of anti-MBP antibodies in the development of EAE induced with MBP [119]. Later studies with μMT (B cell deficient) mice showed that B cells were not required for induction of disease when the mouse MOG_{35-55} antigen was used to immunise but were required for induction if intact recombinant human MOG protein was used to immunise [120, 121]. Differences in amino acid sequences between

human and mouse MOG protein may account for differences in B cell recognition and therefore differences in disease induction.

Macrophages are particularly well adapted to endocytosis but their low expression of MHC class II and costimulatory molecules makes them inefficient at activating naïve T cells. However these can be up-regulated by stimulation with cytokine or bacteria [118]. Macrophages may therefore be key APC at effector sites during infection and inflammation.

The term dendritic cell was first coined by Steinman and Cohn in 1973 [122]. These cells can be considered the 'professional' APC because antigen presentation for the activation of T lymphocytes appears to be their primary function and, although other APC can help to magnify an ongoing T cell response, only DC have the ability to initiate new T cell responses.

They are generated from either myeloid or lymphoid bone marrow progenitors and exist in two main forms, immature and mature. In order for a T cell immune response to be initiated, the T cell (which circulates in blood) must find and recognise antigen (foreign or self) from anywhere in the body; it must also undergo expansion. However the majority of infected tissues are unable to provide the Signal 2 required for expansion [123].

Immature DC (such as Langerhans cells) act as sentinels in the peripheral tissues, such as the epidermis, where antigen is most likely to be encountered [124]. Immature DC express very little in terms of MHC class II and co-stimulatory molecules. However they do express a large number of antigen-capturing Fcγ and Fcε receptors [125] as well as C-type lectins which mediate endocytosis. The combination of antigen capture and macropinocytosis is so efficient that nanomolar concentrations of antigen are enough to elicit a response [126]. DC activation (for example by toll like receptor -TLR- ligation by pathogen-derived molecules) leads to their maturation to potent APC (i.e. increased MHC Class II and costimulatory molecule expression). These mature DC travel to the T cell areas of the lymphoid

organs where they release chemokines attracting T and B cells [127]. They then present antigen derived peptides to T cells to initiate T cell activation [128].

DC are not restricted to interactions with T cells and are known to interact with both NK cells as well as B cells. There are many roles assigned to DC, some opposing and too diverse for the same cell to carry out. There needs to be different subsets of DC which carry out the different roles and there are two models which can be used to explain the generation of these subsets. The functional plasticity model suggests that all DC are members of the same haematopoietic lineage but the 'plastic' end product cell can be polarised into different subsets depending on the immediate milieu. The specialised lineage model suggests that subsets are the result of a lineage separation at the haematopoietic stage and subsequent divergences in the development pathway [129].

Distinct DC subsets were originally defined based on the expression of CD11c (the integrin α_X chain). However more recently these subsets have been defined as myeloid DC (mDC), plasmacytoid DC (pDC),CD8α^+ DC and Langerhans cells [129-131].

mDC have a monocytic morphology and are know to express markers such as CD13, CD33, CD11c and CD11b (the integrin α_M chain of Mac-1) [132] and produce large amounts of IL-12 hence promoting Th_1 cells [133].

pDC are so named because their morphology is similar to B lineage antibody producing plasma cells. These DC are B220$^+$, CD11c^{lo}, Gr-1, 120G8, CD4^{hi}, CD62L and CD123 [131, 134]. pDC were also known as interferon producing cells (IPC) because of their production of IFN-α and IFN-β [131, 135] alongside IL-6 [129].

1.4.3. T Cell Receptor Signalling

Following TCR engagement of a relevant peptide-MHC Class II complex, conformational changes occur between the TCR and CD3 molecules (Figure 1.2). The Src family leukocyte-specific protein tyrosine kinase (Lck) phosphorylates the immunoreceptor tyrosine-based activation motifs in the TCR-associated CD3ζ chains with family member Fyn. This establishes a binding site for the Src homology (SH2) domain of the tyrosine kinase Zap-70 (ζ-chain-associated protein kinase of 70Da). Lck then phosphorylates and activates Zap-70 [136].

In turn this activated Zap-70 phosphorylates the scaffold protein LAT (linker of activated T cells) and SLP-76. LAT provides a surface for the interaction of signalling components responsible for the propagation and dissemination of the T cell signal. Around the same time growth factor receptor-bound protein 2 (GRB2) binds via its N-terminal SH3 domains to the Son of Sevenless (SOS) guanine nucleotide exchange factor (GEF). Along with phospholipase C-γ1 (PLC-γ1), the GRB2 / SOS complex are subsequently recruited to the membrane linked LAT where they are activated by SLP-76 (SH2-domain-cointaining leukocyte protein of 76Da) [137].

The GRB2 / SOS is used by Ras-GDP to allow a guanine nucleotide exchange to convert to its active form Ras-GTP. Once Ras is activated it recruits and activates Raf-1 (serine/threonine kinase) which in turn phosphorylates and activates MEK (MAPK/Erk kinase) [138]. This tyrosine/threonine kinase in turn phosphorylates and activates extracellular signal related kinases 1 and 2 (Erk1 and Erk2). These kinases initiate a MAP (mitogen-activated protein) kinase cascade which induces and activates Fos, a component of the AP-1 transcription factor [139].

PLC-γ1 is activated by SLP-76 to cleave the phosphatidylinositol-4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositoltriphosphate (IP3) [140]. IP3 acts on the receptors on the endoplasmic reticulum to allow an increase in intracellular calcium concentration. The effect of this is activating calmodulin which activates the

serine phosphatase calcineurin. Calcineurin dephosphorylates the NF-AT (nuclear factor of activated T cells) transcription factor. In association with calcium, DAG activates protein kinase C (PKC) isozyymes including PKC−θ which activates the nuclear factor kappa B (NF-κB) transcription factor. DAG also recruits the RASGRP (RAS guanyl-releasing protein) nucleotide exchange factor which helps activate the ERK pathway and therefore activates the AP-1 transcription factor [141].

AP-1, NF-AT and NF-κB act together to induce gene transcription which leads to cell proliferation and differentiation. [142]

The signalling events above can be regulated by various protein tyrosine phosphotases. The first of these to have been identified was CD45 [143] which rapidly activated Lck in T cells by dephosphorylating its negative-regulatory site [144]. CD45 has many other positive effects on signalling including the activation of phospholipase C and mobilisation of calcium [145, 146].

Tyrosine phosphatase-2 (SHP-2) and LMPTP also are known to have a positive regulatory effect on T cell signalling. SHP-2 is known to enhance the activation of both RAS and MAPK [147]. LMPTP dephosphorylates the negative-regulatory site of Zap-70. If this site is allowed to be phosphorylated then it becomes a binding site for CBL (casitas B-lineage lymphoma) E3-ubiquitin-ligase complex which causes the internalisation and degradation of TCR – thereby downregulating TCR signalling (discussed further in Section 1.5.2) [148]. Other E3 ubiquitin ligases such as SLIM (STAT-interacting LIM protein) [149] and Deltex1 [150] have been shown to negatively regulate T cell signalling whereas positive regulation is seen with E3 ubiquitin ligase TRAC1 (T cell RING protein identified in activation screen 1) [151].

LAT has also been shown to interact via GRB2 with the adaptor protein GRB2-associated binding protein 2 (GAB2). This enables the recruitment of tyrosine phosphatase-1 (SHP-1) which in turn down-regulates T cell activation by dephosphorylating Zap-70 [152]. Another molecule, CD5, which negatively regulates signalling, is discussed in greater detail in Section 1.5.2.

Figure1.2

1.5. T Cell Tolerance

The number of different peptides that can be generated and presented far outweighs both the number of different TCR that gene segment recombination allows and the total number of T cells within a mouse. As a result if individual T cell recognised only one peptide, we would still be left short. This inequality in numbers leaves no option other than to have a cross-reactive TCR repertoire; otherwise the immune system may not recognise the many pathogens that can infect the host. Even with this TCR promiscuity Don Mason predicted that each naïve $CD4^+$ cell must have the capacity to react with almost half a million different 11-mer peptides [153, 154].

This 'cross-reactivity' has a flipside; in maintaining its ability to mount an effective response to a wide range of foreign antigens the TCR also retains the ability to react to a wide range of self antigens. This is why the immune system must eliminate self-reacting T lymphocytes [155]. It must learn to be 'self-tolerant'. The failure of tolerance can often lead to autoimmune pathology.

1.5.1. Central Tolerance

Kappler *et al* showed self–reactive T cells were present amongst immature thymocytes but were 'severely depleted' amongst mature thymocytes, hence showing that clonal elimination occurs in the thymus [156]. This elimination is termed 'central tolerance' and serves as the primary route of limiting the cross-reactivity of the T cell repertoire.

The mechanisms behind central tolerance and the selective pressures on T lymphocytes during thymic development are thought to be based on an avidity model of T cell activation. Avidity is described as the number of peptide-MHC (pMHC) complex engaged TCR per T lymphocyte. The primary factor determining avidity is the intrinsic affinity of the TCR for the pMHC complex. The density of the TCR and the density of the pMHC complexes are also factors [157]. The functional T cell avidity can therefore be defined as the concentration of peptide that leads to half the

maximal activation of T cells in a given population for a constant number of APC [158]. The peptide concentration at which T cell activation occurs is suggested to be inversely proportional to the affinity of the TCR.

Negative selection results from high avidity interactions with self pMHC complexes and involves TCR mediated apoptosis [159], whereas low avidity interactions with self pMHC complexes promote survival of naïve immature thymocytes (positive selection) [159]. Alternatively the self-reactive TCR can be edited by V(D)J recombination to display a less reactive TCR, or TCRs can be down-regulated and activation inhibitory receptors can be up-regulated (such as CTLA4) in response to strong stimulation in the thymus [160].

Liu and colleagues examining central tolerance relevant to EAE, found that the administration of MBP_{Ac1-9} in PBS to Tg3 mice, via the intraperitoneal route, resulted in unresponsiveness of T cells [49]. Further examination of the model showed that a 77% reduction of $CD4^+CD8^+$ cells in the thymus occurred within hours of administration, indicating that the mechanism of tolerance is deletion.

1.5.2. Peripheral Tolerance

Central tolerance is not a perfect system however, and some self-reactive T cells can escape clonal elimination and therefore must be controlled via peripheral mechanisms in order to avoid harm.

There appear to be three key principles currently suggested concerning peripheral tolerance to self and administered antigen; deletion, regulation and anergy-adaptation. These may not be exclusive and it is possible that a T cell may experience the latter two before going on to be deleted [161].

The consensus view is that peripheral tolerance is maintained by self-antigen presentation by steady state (the 'absence of deliberate exposure to maturation signals') immature DC in the absence of Signal 2 [162].

Deletion

Peripheral T cells can die by either extrinsic or intrinsic mechanisms. Death in the absence of appropriate cell survival signals is also known as death by neglect or activated-cell autonomous death (ACAD) [163]. Death by excessive stimulation of an already activated cell is termed activation – induced cell death (AICD) [164]. Either of these fates are determined by the signals received by the TCR and although both have different initiating events, both pathways converge at the caspase (proteolytic enzymes) level to finalise death. In an extrinsic mechanism of death pro-apoptotic signals are mediated through the production of a death-inducing signal complex (DISC) [165]. The intrinsic mechanism of death relies on the interplay between pro and anti-apoptotic members of the B cell lymphoma 2 (Bcl-2) family [166].

The first observation of cell death via apoptosis was made by Ochi *et al*; they showed that a challenge with superantigen resulted in clonal expansion of relevant $CD4^+$ T cells followed by a dramatic loss of the same cells to a level lower than in the naïve mouse [167]. DNA fragmentation was detected in the population of stimulated cells for a limited time after this activation; hence the cells were shown to have undergone apoptosis as a result of their activation.

ACAD

The intrinsic apoptotic pathway may be a result of either neglect (cytokine deprivation) or stimuli such as UV radiation, DNA damage and endoplasmic reticulum stress. It is dependent on the actions of the Bcl-2 family members, BIM (Bcl-2-interacting mediator of cell death) and PUMA (p53-upregulated modulator of

apoptosis) [168]. Bcl-2 helps to regulate mitochondrial activity and once BIM and PUMA bind to Bcl-2 the regulation is disrupted and cytochrome c is released. This pathway is therefore a result of the permeabilisation of the mitochondrial membrane and the release of cytochrome c aids the formation of the apoptosome. Caspases begin to work at the apoptosomal level.

AICD

The TCR restimulation of already activated and expanded T cells in the absence of signal 2 can lead to AICD [169]. Studies have used Fas (CD95) deficient mice to implicate the Fas pathway within AICD. Other studies have implicated TNF receptor 1 (TNFR1) [170] and TNF related apoptosis-inducing ligand receptor (TRAILR) [171]; the latter in $CD8^+$ cells.

AICD was shown to be a result of the upregulation of FasL, which induces Fas-mediated death of either the same cell on which FasL is expressed (suicide) or on a neighbouring cell. The pathway is dependent on the interaction between Fas and FasL; the upregulation of both markers on activated T cells leads to the activation of a CD95-DISC complex which initiates further downstream events mediating apoptosis of these cells [172-174]. It is the presence of IL-2 and its effects on several transcription factors including NF-AT and NF-κB [175] that determines Fas and FasL expression respectively and explains why Fas in not largely expressed on naïve cells. During the course of normal disease AICD appears to limit pathology by preventing the over-expansion of relevant T cell populations when antigen is continually being presented to the cells [176].

Regulation

As early as 1969, Sakakura and colleagues [177] showed that thymectomy of mice at day two of life led to autoimmune ovary destruction. Evidence has since gathered to

show that peripheral tolerance is aided by nTregs that have been selected for in the thymus. These cells have higher avidity than positively selected T cells but lower avidity than clonally deleted cells [178, 179].

Foxp3$^+$ Regulatory Cells

Foxp3$^+$ Regulatory Cells make up around 10% of the normal CD4$^+$ T cell population in mice [180]. These cells are found to co-express CD25 (IL-2 receptor α chain) [181] and have been shown to control the expansion of self-reactive cells. However the nTreg population has only recently been fully distinguished from activated CD4$^+$ T cells expressing CD25 by the expression of the X-chromosome-encoded forkhead transcription factor, Foxp3 [182, 183]. Foxp3 mRNA was found to be present mainly in CD4$^+$CD25$^+$ cells [184]. CD25$^-$ cells found to express Foxp3 were CD45RB^{lo} and these cells are implicated in regulation also.

CD25$^{-/-}$, as well as IL-2$^{-/-}$ Tregs are able to suppress T cell proliferation in vitro suggesting that IL-2 is not required for suppression to occur. The existence of a Foxp3$^+$ population of nTregs in these mice also indicated that IL-2 is not essential in thymic selection of nTregs however IL-2 signalling has been shown to be a requirement for cell growth and metabolism [185].

The Foxp3$^+$ population of cells express the CD25 marker regardless of activation state alongside expression of CTLA-4, glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR) [186], and a minority express CD103 (α_E integrin) [184].

Adoptive transfer or CD4$^+$CD25$^-$ cell populations into athymic mice results in the development of an array of autoimmune diseases. Transfer of CD4$^+$CD25$^+$ T cells shortly after CD4$^+$CD25$^-$ transfer prevents autoimmune pathology in cell dose-dependent manner [181]. Tregs require TCR ligation by specific antigens in order to

exert regulatory effects; this suggests that Tregs need to be self-reactive to some degree to allow control of other self-reactive cells [187].

Evidence supporting this came from Rudensky and colleagues [188]; significant diversity of $CD4^+$ $CD25^+$ and $CD25^-$ T cell repertoires was established through sequence analysis. The specificity of cloned Treg TCR was then analysed using retroviral expression in mono-specific TCRαβ transgenic recombination-activating gene knock-out ($RAG^{-/-}$) T cells. This approach showed that a large proportion of $CD25^+$ TCR were more effective in recognising constitutively presented self-antigens, compared to the $CD25^-$ repertoire [188].

nTregs are thought to mediate their effects through IL-10 and TGF-β1 production [98, 189]. IL-10 production is not dependent on the expression of Foxp3 or CD25. However chronic antigenic stimulation is known to result in $CD25^+$ cells; these T_R1 cells produce IL-10 and appear to be as efficient as nTregs in suppressing disease [190, 191]. Both nTregs and T_R1 cells are shown to decrease IL-2 production to similar extents [191].

T Regulatory 1 (Tr1) Cells

The first identification of Tr1 cells was by Groux and colleagues; these OVA-specific transgenic cells were generated experimentally *in vitro* by chronic stimulation with IL-10 and OVA peptide [98]. These cells can be distinguished from Th_1 and Th_2 cells by their secretion of high levels of IL-10 as well as secreting low levels of IL-2, IL-4, IL-5, TGF-β and IFN-γ [98]; the levels of secretion of cytokines (except IL-10) depend on the model in which the Tr1 cells are induced. Tr1 cells have also been isolated from human peripheral blood. Tr1 cells express the IL-2 and IL-15 receptor α chains in conjunction with either the receptor β or γ chains of both cytokines [192].

In an EAE model of epitope spreading, the administration of a spreading determinant ($MBP_{68–86}$), in rats immunised with $MBP_{87–99}$ and CFA activated Tr1 cells and potentiated their expansion in the cervical lymph nodes and spleen. The antigen relevant production of IL-10 from these cells was implicated in the bystander suppression of disease; the administration of anti-IL-10 antibodies abrogated the effect of Tr1 produced IL-10 [193].

T Helper Type 3 (Th_3) Cells

Oral tolerance is of importance in immunology because it is a continuous natural immunological event which is driven by exogenous antigen. Within EAE this was first shown in the Lewis rat with high doses of MBP by Bitar *et al* and multiple low doses of MBP by Higgins *et al* 1980s [194, 195]. Later work revealed that the mechanism of tolerance in high dose administration was anergy [196] whereas in low dose administration it was via transferable cellular suppression [197].

T cell clones from orally tolerised mice have been isolated and have been defined as the unique set of $CD4^+$ cells, Th_3 [198]. These cells are MHC class II restricted and share the same αβ TCR as Th_1 and Th_2 cells [189]. They appear to require IL-4 rather than IL-2 for growth and some clones produce low level amounts of IL-10 and/or IL-4 but no IFN-γ or IL-2 upon TCR ligation [198].

The Th_3 repertoire is thought to primarily exert its effects through the production of TGF-β1 upon TCR ligation. They are thought to mediate TGF-β suppression through a cell-contact dependent mechanism relying on surface bound TGF-β [189]. Studies have shown TGF-β1 dependent suppression of $CD8^+$ T cells in type 1 diabetes [199] and tumour environments [200] as well as suppression of $CD4^+$ mediated EAE.

Regulation in EAE

The role of B cells in regulation was suggested by Janeway and colleagues who reported that μMT mice on the H-2^u background failed to recover from EAE [201]. The same effect was seen by Fillatreau and colleagues in μMT mice on the H-2^b background immunised with MOG_{35-55} [99]. Adoptive transfers of B cells from recovered mice into recipient naïve mice have shown protection against subsequent disease induction. IL-$10^{-/-}$ mice show the same lack of disease remission as μMT mice [97] and on closer examination the effector or memory B cells taken from mice recovered from EAE have shown secretion of IL-10 when stimulated through BCR and CD40 [202].

B cells from MS patients produce a decreased amount of IL-10 compared to non-sufferers suggesting that these cells have a role in regulation or limitation of MS onset [203]. Recent observations show that B cells can also be stimulated to produce IL-10 through TLRs 2 and 4. The B cells however are unable to mediate suppressive effects on activated T cells by themselves; the B cells affect the production of IL-6, IL-23 and TNF-α from DC through IL-10 dependent mechanisms [204]. Despite these findings showing the necessity of B cells in the recovery of EAE; kinetic studies show that B cells do not accumulate in the CNS, in significant numbers, at any point during a course of the disease.

Recovery of MOG_{35-55} induced EAE, in C57BL/6 mice, correlated with an accumulation of $CD4^+CD25^+Foxp3^+$ T cells only within the CNS. One in three CNS $CD4^+$ cells were Tregs and from this population one in three could produce IL-10 (around 10% of the total $CD4^+$ CNS infiltrate). The depletion of these cells impaired recovery and the transfer of the same population back into sick mice ameliorated disease [205]. Treg depletion was also seen to exacerbate $PLP_{139-151}$ induced EAE. The same series of experiments identified a role for TGF-β by showing that anti-TGF-β treatment prevented recovery from disease [206].

These data suggest that B cells play a role in preventing the expansion of T cells with pathogenic potential in the peripheral lymphoid organs but it is the Tregs that are the dominant force of regulation within the CNS.

Functional Inactivation of Lymphocytes

Anergy is the term coined for a situation where lymphocytes are functionally inactivated following an encounter with an antigen. For lymphocytes to be 'anergic' they must be hypo-responsive and also must stay alive for much longer than the 24 hours reflective of cells in an apoptotic state [207]. Anergy is currently categorised into two types, clonal anergy and adaptive tolerance. Schwartz suggests that clonal anergy is a phenomenon of cell cycle arrest in activated T cells whereas adaptive tolerance (or adaptation) is a result of persistent stimulation of naïve T cells [207]. Lechler *et al* showed that the conditions that led to the non-responsiveness of activated T cells, the ligation of the TCR without full co-stimulation, failed to anergise naïve T cells [208].

A likely cause for clonal anergy appears to be the failure of a CD28-B7 interaction whilst the TCR is being ligated leading to an unresponsive cell. CD28 signalling down-regulates the cell cycle inhibitor $p27^{kip1}$. In the absence of CD28 signalling, $p27^{kip1}$ is able to bind to and block the actions of cyclin E-Cdk2 in late G1. E-Cdk2 is required to phosphorylate anti-proliferative transcription factors Smad 2 and Smad 3 and hence allows entry into S phase and induces production of IL-2; IL-2 itself then acts to down-regulate $p27^{kip1}$ [209, 210]. CTLA-4 may act to inhibit CD28 signalling and hence induce anergy [208].

The presence of Rapamycin can also induce anergy, even with CD28 ligation and the production of IL-2 by activated cells. Rapamycin exerts its effects by inhibiting the activation of mTOR, a protein kinase required for cell division [207, 209].

Recent studies have shown a defect in LAT phosphorylation and palmitoylation in anergic cells. As LAT is the primary site where key molecules such as GBR2, SLP-

76 and PLCγ1 come together this has obvious effects on the ability of a TCR signal to be propagated [211].

The uncoupling of Ras from the TCR in anergic cells has also been cited as a key event in maintenance of anergy. Ras binds GTP even in anergic cells to form Ras-GTP, however the problem appears to be the constitutive expression of GTP-activating protein (GAP) which converts Ras-GTP into the inactive form Ras-GDP [207]. Ras-GTP activates Raf-1 however this can be prevented by the actions of Rap1-GTP. The action of diacylglycerol (DAG) to lead both Ras-GTP and Raf to the immunological synapse appears key to the MAP kinase and ERK signalling pathways respectively. The excessive production of DAG kinase in anergic cells leads to loss of DAG and hence impairs both signalling pathways; this in turn dampens transactivation of the AP-1 transcription factor which mediates the production of IL-2 [212]. Importantly it has been also noted that the constitutive production of Ras in anergic Th_1 clone restores IL-2 production and MAPK activity [213].

The ubiquitylation pathway is used to target a protein for destruction by the 26S proteosome. It consists of a core molecule, Ubiquitin, enzymes involved in ubiquitin attachment (ubiquitin ligases E1, E2 and E3), deubiquitylating enzymes and ubiquitin-binding proteins. The E3 ubiquitin ligases that have been identified in relation to anergic T cells include CBL-B [214], GRAIL (gene related to anergy in lymphocytes) [215] and ITCH (itchy homologue E3 ubiquitin protein ligase).

CBL-B prevents the association of CD28 and phosphoinositide 3-kinase by polyubiquitylating the p85 subunit of the latter. This inhibits the signal 2 cascade. CBL-B and GRAIL inhibit actin reorganisation which is required to allow the formation of the immune synapse. GRAIL mediates this by polyubiquitylating and stabilising the RHO-guanine-dissociation inhibitor. ITCH monoubiquitylates PLC-γ1 and PKC−θ and therefore can have an impact on the calcium signalling pathway , the DAG pathway and the PKC pathway which modulate NF-AT, AP-1 and NF-κB respectively [216].

Adaptive Tolerance

Adaptive tolerance is essentially unresponsiveness to persistent antigen stimulation with the key being that the cell can return to a functional phenotype once the stimulus is removed; that is to say, the state is reversible. This system is beneficial in limiting T cell mediated damage to host after a pathogen is cleared from the system. Adaptive tolerance can be explained in part by the tuneable activation threshold (TAT) model postulated by Grossman and Paul [217]. This model explains the modulation of T cells in response to antigenic stimulation; theorising that T cells maintain tolerance to self-MHC-peptide complexes by tuning their TCR signalling to match the background level of stimulus. A rapid change in the level of stimulus will elicit a response from the T cells however if this new stimulus is constant or recurrent, then the T cell will adapt accordingly by readjusting its activation threshold [218, 219] to once again being tolerant to this new stimulus. Hence the T cell remains functionally unresponsive to stimuli of strengths equal to or below that of the initial persistent stimulus [161].

Schwartz and colleagues created a model of adaptive tolerance where they were able to exclude the problems of studying relevant T cell repertoires in a heterogeneous environment. $RAG^{-/-}$ TCR transgenic $CD4^{+}$ mice specific for pigeon cytochrome C (PCC) were crossed with a second transgenic mouse which expressed PCC under the control of an MHC class I promoter and an Ig enhancer. This second mouse had already been crossed with a $CD3e^{-/-}$ mouse and therefore had no T cells. The final mouse had a large monospecific adapted T cell population with minimum deletion due to the low expression of antigen. If cells from this mouse were transferred into a host not presenting PCC then they regained responsiveness however if transferred into a host with PCC the cells initially regained responsiveness but then settled into a deeper state of adaptation than before. The intrinsic adaptive tolerance of the $CD4^{+}$ cells in this mouse could be induced to different levels. These observations showed the reversibility of the adaptive tolerogenic state [220].

Various differences exist between anergic and adapted cells. The main difference appears to be at the level of T cell signalling impairment. As discussed earlier, the

Ras / MAP kinase pathway is inhibited in anergy whereas Zap-70 appears inhibited in adaptive tolerance, leading to disruption of the calcium and NF-AT pathways [221]. Furthermore in adapted cells the blocked proliferation cannot be reversed by adding IL-2. Clonally anergic cells remain inactive for long periods of time after the antigen and APC are removed whereas adapted cells appear to regain responsiveness relatively quickly [207].

As discussed previously, the number of potential antigens to recognise far outweighs the potential number of T cells that are available to the immune system. With that in mind although the autoaggressive potential of T cells needs to be controlled, death may not be the perfect answer because some of the T cells that die may be required to detect non-self antigens. Adaptation appears to be the perfect solution; the dynamics of T cell adaptation suggests that auto-reactive T cells can be maintained in the peripheral T cell repertoire with the potential to react against foreign invaders which stimulate them greater than cognate antigen. Hence the immune system can maintain a more diverse T cell repertoire.

The Role of CD5

Cells seem to be adapted in part through the actions of a monomeric cell surface glycoprotein, CD5, which appears to negatively regulate TCR signalling. CD5 is constitutively expressed on T cells, B cells and thymocytes and is known to be associated with the TCR / CD3 complex at the T cell / APC interface. Its recruitment appears to be at a similar rate to CD3; its role however does not appear to be linked to the formation or stability of the immunological synapse. Data comparing CD5 high, low or negative cells indicates that levels of CD5 correlate with an effect on the antigen induced tyrosine phosphorylation in T cells affecting the proliferative potential of these cells [222].

After the TCR is engaged, CD5 becomes tyrosine phosphorylated and forms a complex with molecules such as CD4 and Zap70 [223, 224]. Reduced CD5 levels are

shown to correlate with increased Ca^{2+} responses to TCR engagement [76, 225]. The exact mechanism by which CD5 affects signalling and whether it acts alone as a negative regulator of TCR is unclear as is the question of which other factors are involved in desensitising T cells.

Ryan *et al* suggested that T cells can avoid Fas-mediated AICD because of a functional desensitisation that correlates with enhanced CD5 expression [174]. This was also implicated in lung carcinoma studies; incubation of T cells with an anti-CD5 antibody resulted in a 4-fold increase in surface expression of FasL without showing increase in FasL mRNA levels. This would indicate that CD5 was preventing the delivery of FasL to the cell surface by manipulating TCR signalling. The level of control at which CD5 can manipulate AICD may be through Caspase-8, a key molecule in the in the Fas-associated death domain [226].

This supports data mentioned above suggesting that CD5 was manipulating Zap-70; it has been shown in separate studies using NF-ATc2 and c3 deficient mice that there is a loss of FasL expression and impaired AICD hence the manipulation of Zap-70 would affect the calcium signalling which activates the NF-AT transcription factors [227].

1.6. Breaking down tolerance in autoimmune disease

1.6.1. Molecular Mimicry

A single T cell has the ability to respond to both infectious agents and self tissue because of the cross-reactive nature of the TCR. The molecular mimicry theory, within autoimmune disease, suggests that an immune response initially directed at a non-self antigen by a T cell can spread to a self antigen because of the cross-reactivity of the TCR. Initial experiments compared hepatitis B with MBP and data suggested that foreign and self antigens had to share sequence stretches of at least 6 amino acids in order to initiate expansion of cells relevant to the other [228, 229].

Using a model of insulin-dependent diabetes mellitus (IDDM), Oldstone and colleagues later showed molecular mimicry at work; either the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) or the LCMV nucleoprotein (NP) were transgenically expressed in pancreatic beta cells in mice. The data showed that the low expression of these viral gene products rarely produced a case of IDDM, however if mice were subsequently challenged with LCMV then an immunological attack on the pancreas results and several aspects of IDDM were observed. Although initial work was carried out with virions, this is not a limit to the potential of molecular mimicry [230].

Ohashi *et al* combined these findings with mice bearing transgenic TCR for the LCMV GP. This allowed the tracking and evaluation of a defined repertoire of antigen-relevant cells in H-2^{Db} transgenic mice. Results showed that tolerance in this model was not a result of clonal deletion, clonal anergy nor a modulation in cell surface markers and yet the cells showed a sustained period where they remained unresponsiveness [231].

Sequence similarities between MBP and viral peptides have been indicated in database searches and *in vitro* testing of MBP relevant human T cell clones has shown that some of these clones showed reactivity to some of the viral peptides [232]. Greater clarification of the binding of antigen to the TCR and the MHC class

II molecule involved led to the discovery that cross-reactivity was determined by key residues within the antigen sequences that were embedded in each receptor [232, 233]. The identification of these contact motifs indicated a more frequent cross-reactivity in T cell recognition than previously thought.

Using the EAE model, Fujinama and Oldstone showed that immunisation of rabbits with the hepatitis B viral polymerase induced CNS inflammation but not active disease [229]. Similarly, sequences homologous to the myelin PLP found in murine hepatitis virus and *haemophilus influenzae* type B could stimulate $PLP_{139-151}$ relevant cells but could not actively induce disease; although they did enhance disease when PLP was subsequently administered [54].

More recent work showed that a MBP_{87-99} relevant TCR could bind a sequence that differed from the MBP_{87-99} sequence in all 13 amino acid positions. This would negate the theory that shared amino acid anchor residues between sequences are required and indicate that each amino acid within a sequence contributes to the overall affinity of the MHC-peptide complex to the TCR [234]. The TCR would seem far more promiscuous than initially thought [235].

1.6.2. Escape from Central Tolerance In EAE

Central tolerance is not a perfect system and some EAE models can help to explain how certain auto-aggressive T cells end up within the peripheral repertoire.

The $PLP_{139-151}$ epitope is the immunodominant epitope of PLP within H-2^s mice and yet the frequency of $PLP_{139-151}$ reactive $CD4^+$ cells within the SJL strain is as high as 1/20,000 $CD4^+$ cells. The gene which encodes PLP is alternatively spliced to produce the DM20 isoform which lacks the 116-150 sequence and therefore also the $PLP_{139-151}$ epitope. This isoform is preferentially presented in thymic selection, despite both DM20 and PLP being presented within the CNS, and therefore allows the escape of relevant cells from negative selection into the periphery because the $PLP_{139-151}$ epitope is either absent or present in very low levels [42] [236].

The immunodominant epitope in H-2^U mice, MBP_{Ac1-9}, is notable for its immeasurable binding affinity to the A^u MHC class II molecule. It is the lysine at position 4 of the sequence that is to blame for the poor MHC binding as it interacts unfavourably with the hydrophobic pocket in the I-A^u binding groove [237] [238]. An avidity based model of selection in the thymus suggests that Ac1-9 relevant T cells may be allowed to survive due to poor interaction with the presented antigen [49]. This can be used to explain the escape of a high frequency of MBP_{Ac1-9} relevant T cells bearing high affinity TCR into the peripheral T cell repertoire.

1.7. Peptide Induced Therapeutic Tolerance

Peptide alone cannot induce EAE, hence the use of adjuvant to provide the 'danger' signal which helps to induce disease. The presentation of antigenic peptide in the absence of co-stimulatory molecules has long been known to cause T cell tolerance through anergy [239] due to the absence of Signal 2. If harnessed for therapy, peptide induced tolerance may be preferential to other forms of therapy because it allows for the manipulation of only the disease relevant cells and with the various routes in which peptide can be administered the level of control exerted upon the immune response can be more closely monitored and adjusted.

There are four different protocols currently used to facilitate the induction of peptide mediated therapeutic tolerance; soluble-peptide induced tolerance, mucosal (nasal or oral) induced tolerance, peptide coupled cell induced tolerance and altered-peptide ligand (APL) induced tolerance.

Soluble Peptide Induced Tolerance

Soluble-peptide induced tolerance has previously been successful in the prevention of disease in models of type 1 diabetes [240] and several groups have shown EAE induction in mice is suppressed when soluble encephalitogenic MBP epitopes are administered intraperitoneally and intravenously (i.v.) without adjuvant [241, 242]. Low doses of peptide are shown to result in an immune response whereas a high dose results in tolerance – deletion [243] and anergy [244].

Hilliard [245] and others [246] have showed that the i.v. administration of peptide after onset of disease leads to clonal deletion of cells via apoptosis whereas administration of peptide just prior to or just after immunisation results in antigen-specific regulation leading to tolerance. This group has also shown that the tolerance induced by i.v. administration of peptide results in the suppression of IL-12 [246].

High-dose peptide treatment in mice with an established course of EAE has however been known to induce a fatal anaphylactic response in some murine strains [247] and i.v. MOG administration in primates has been shown to intensify disease [248].

Mucosal and Peptide-Coupled Cell Induced Tolerance

Mucosally induced tolerance is based on the introduction of large numbers of foreign antigen into the body, via food, and the ability of the immune system not to react against them, except in the case of food allergies. Mucosal tolerance is advantageous because of the ease of administration and a decreased risk of toxicity compared to injection of antigen.

Mice with expanded DC populations, after a single feeding with MBP, were shown to be tolerant to EAE induction potentially due to quiescent DC; DC that had been stimulated with LPS did not confer tolerance. Tolerance inducing DC also appeared to have up-regulate the production of IL-4, IL-5 and IL-10 thus indicating a bystander regulation mechanism.

High dose oral antigen has been shown to induce anergy or deletion of antigen relevant cells [194, 241]. Antigen is absorbed through M cells in the Peyer's patch and is delivered via the bloodstream to the spleen or lymph nodes where it is detected and taken up by DC. The DC can then process and present the antigen to circulating T cells [249]. DC induce tolerance in T cells after the induction of signal 1; instead of T cells slowing down and allowing longer contacts through DC signalling, resulting in priming, the DC appear to no longer provide these signals and the T cells remain dynamic leading to tolerance [250]. Low dose oral antigen tolerance is based on the induction of Th3 cells [198] or by the induction of bystander suppression.

Within EAE, attempts to prevent the induction of disease with prior nasal or oral administration has been successful [251, 252] however treatment of pre-established disease has only been successful using a combination of antigen and soluble IL-10 [253].

If peptides given orally can induce tolerance then the implications for autoimmune disease are clear. Unfortunately clinical trials have thus far been unsuccessful [18].

Peptide coupled cell induced tolerance relies on the use of ethylene carbodiimide fixed splenocytes coupled to the relevant antigen. The intravenous introduction of cells coupled with PLP antigens has been reported to prevent the induction of EAE [254]. There are two mechanisms that are suggested to explain the actions of these peptide coupled cells; sub-optimal activation of T cells in the absence of signal 2 or the induction of apoptosis of the coupled cells and the re-presentation of their peptides in a non-inflammatory manner by macrophage.

Altered Peptide Ligand Induced Tolerance

Evavold *et al* originally used the phrase 'altered peptide ligands' to describe peptides that had individual amino acid substitutions to define residues important in interacting with TCR or MHC molecules [255]. Today the term incorporates peptide

sequences with multiple amino acid substitutions. APL are able to be used to manipulate the TCR signalling cascade because they can compete for the same TCR binding sites as wild-type antigens. Single or double amino acid substitutions within the relevant sequences allow APL to retain a certain degree of agonistic potential. Sub agonist APL are still able to activate T cells but to a lesser degree than the wild-type peptide, whereas superagonist APL activate T cells to a greater extent than the wild-type.

Initially, experiments by Wraith and colleagues used these APL to establish the key residues for the binding of MBP_{Ac1-9} to both the MHC Class II molecule and the TCR. Substitutions in the wild type (4Lys) sequence showed that residues 3 and 6 were critical to TCR binding whereas 4 and 5 were critical to MHC binding [256, 257].

In later EAE experiments, Smilek and Wraith [258] used a variation of the MBP Ac1-9 epitope to modulate disease course - 4Ala (substitution at position 4, replacing the Lysine with Alanine). Whereas 4Lys immunisation gave a standard course of disease, co-immunisation with 4Ala prevented disease. This data suggested that, although the T cells may not have reacted to the APL, they were certainly aware of its presence and had changed their behaviour accordingly.

A tyrosine substitution allowed further manipulation of disease course through mucosal tolerance. Intranasal administration of the 4Tyr APL was shown to suppress disease induced with the 4Lys peptide [259]. This tolerance was shown to be dose dependent; high doses resulted in deletion of cells or anergy, whereas low doses induced regulatory T cells which down-regulated the disease-relevant cells. In this series of experiments, 3 key facets of the APL were elucidated: antigenicity *in vitro* correlates to tolerogenicity *in vivo*; tolerogenicity does not correlate to immunogenicity; antigenicity *in vitro* does not correlate to immunogenicity *in vivo* [259].

Work on T cell lines (TCL), generated against APL, using B10.PL mice showed that cells responded to the priming APL at nM concentrations; however as the superagonist properties of the APL increase there is an associated loss in their capacity to react against the 4Lys peptide. This implicated reduced sensitivity of 4Tyr stimulated cells as the cause of an impaired response to 4Lys peptide in the CNS and hence explains the inability of 4Tyr to induce disease [260]. Later work provides strong evidence to suggest that AICD is also responsible for 4Tyr's inability to cause disease [159] and that this is dependent on FAS and FASL expressed on the T cells themselves [174].

In the same disease model, using the Tg4 transgenic mouse and a series of MBP_{Ac1-9} based APL, Ryan *et al* showed that superagonist APL stimulation *in vitro* limited the pathogenic potential of auto-aggressive cells to the wild-type antigen. Two mechanisms were implicated in this tolerance, deletion of some members of the repertoire through Fas mediated apoptosis and adaptation of others [174, 261]. However *in vivo* immunisation with some of these super-agonist APL led to the induction of disease [50].

Steinman and colleagues have developed an APL system based on the 87-99 epitope of MBP and have shown that TNF-α and IFN-γ production from lymph node cells can be reduced when MBP_{87-99} is administered alongside an antagonist APL compared to MBP_{87-99} immunisation alone [262].

In EAE experiments with $PLP_{139-151}$ analogues, results *in vitro* suggested the APL acted as antagonists and *in vivo* suppressed EAE induced with a variety of PLP, MBP and MOG epitopes [263]. These APL were also found to deviate the immune response and T cell clones raised to them were found to produce IL-4, IL-10 and TGF-β [264]. PLP itself has been shown to suppress proliferation responses to MBP peptides when administered intranasally. PLP has also been shown to reduce relapse rates and severity in whole myelin induced disease [259].

1.8. Epitope Spreading

Epitope Spreading is the diversification of the immune response from an initial epitope to a secondary epitope either on the same protein or on a separate protein; it has been suggested that epitope spreading may be responsible for the relapses in relapsing-remitting course of MS.

The epitope spreading paradigm was first observed by Lehmann *et al*, in an EAE model, when disease was induced in the B10.PL x SJL model using MBP_{Ac1-9} [265] and the immune response was seen to change its focus to other epitopes on MBP after an initial Ac1-9 directed response. This observation is now described as intramolecular epitope spreading. Intermolecular epitope spreading is when the immune response spreads from an epitope on one molecule to an epitope on another molecule [266].

As Figure 1.3 shows both these types of spreading are seen in SJL mice immunised with $PLP_{139-151}$, which results in a relapsing–remitting form of EAE. Using T cell proliferation assays it has been shown that, just before and during the first relapse, $PLP_{178-191}$ reactivity occurs (intramolecular epitope spreading) and that MBP_{84-104} responses are seen in the second relapses (intermolecular epitope spreading) [267]. It is suggested that the myelin destruction mediated in the first episode of EAE reveals the $PLP_{178-191}$ cryptic epitope leading to a $PLP_{178-191}$ directed immune response; the first relapse causes further damage to the myelin revealing the MBP_{84-104} cryptic epitope (Figure 1.3).

Hence tissue damage caused in the CNS during an initial phase of EAE may prime a hierarchal cascade of autoaggressive T cells by revealing cryptic epitopes against which several phases of disease are subsequently directed. In this scenario, if epitope spreading is responsible for the relapses in MS then the changes in specificities of the immune response over a period of time could pose problems for single antigen-based immunotherapy. Future therapeutic measures would need the ability to modulate

pathogenic responses to several antigens as immune responses to individual antigens fluctuate.

A flipside to epitope spreading is the phenomenon of bystander suppression; the concept that regulatory cells induced by one antigen can suppress immune responses stimulated by another antigen. This was first described in an investigation of regulatory cells that were induced by oral administration of low doses of MBP (Miller, A. JEM 1991).

Figure 1.3

1.9. Aims of Project

This project had three aims:

1. To develop novel TCR transgenic based models to investigate epitope spreading and adaptive tolerance.

2. To determine the contribution of epitope spreading in $PLP_{139-151}$ induced EAE by tracking and characterising disease relevant pathogenic and regulatory T cells and to assess their contribution on the course of disease

3. To investigate the tuning of the immune response at both a T cell population and individual level in MBP_{Ac1-9} induced disease. To assess the impact of adaptation on the autoreactive T cell repertoire in terms of behaviour and phenotype. We also wished to determine the mechanisms involved in changing T cell sensitivity during adaptation.

Chapter 2:

Materials and Methods

2. Materials and Methods

2.1. Mice

All mice were bred and maintained, under specific pathogen free conditions, at The Institute of Infection and Immunology Research at The University of Edinburgh.

C57BL/6 mice (H-2^b) and B10.PL (H-2^u) mice were used extensively to produce the C57BL/6 x B10.PL F_1 (H-$2^{u,b}$) mouse. SJL (H-2s) mice were used to produce the B10.PL x SJL F_1 mouse. Transgenic strains utilised included the 2D2 [43] mouse, on the C57BL/6 background and the Tg4 mouse on the B10.PL [49] background. CD45.1^+ and CD90.1^+ congenic mice were also used. All mice were sex-matched within experiments and used at 6-10 weeks of age.

2.2. Peptides

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNK), MBP_{Ac1-9} 4Lys (ASQKRPSQR), MBP_{Ac1-9} 4Tyr (ASQYRPSQR) and $PLP_{139-151}$ (HCLGKWLGHPDKF) were synthesised by the Advanced Biotechnology Centre, Imperial College, London using standard F-moc chemistry.

2.3. Adjuvants

Complete and incomplete Freud's adjuvants (CFA and IFA respectively) were obtained from Sigma, Poole, GB. CpG oligonucleotide was obtained from MWG Biotech, London, UK.

2.4. General Reagents

2.4.1.1. Tissue wash buffer:

RPMI 1640 medium containing 25mM HEPES buffer, 2mM L-Glutamine, 100U/ml Penicillin, 100µg/ml streptomycin and $5x10^{-5}$ 2-mercaptoethanol (all from Gibco, Life Technologies, Paisley, UK hereafter referred to as Gibco). 2-mercaptoethanol acts as a reducing agent and improves conditions for cell survival.

2.4.1.2. RPMI-5 tissue culture medium:

Tissue wash buffer with the addition of 5% foetal calf serum (FCS; Sigma; heat-inactivated for 30 minutes at 56°C to denature complement).

2.4.1.3. MACS buffer:

Hanks Balanced Salt Solution with 2% heat-inactivated FCS, 100U/ml Penicillin and 100µg/ml streptomycin (Gibco).

2.4.1.4. FACS buffer:

PBS (sterile; magnesium- and calcium-free; Gibco) supplemented with 2% FCS.

2.4.1.5. Pertussis Toxin (PTx):

PTx was obtained from the Health Protection Agency, Dorset, UK and diluted in PBS.

2.4.1.6. Bicarbonate Buffer (10x)

Na_2CO_3 (6.36g) and $NaHCO_3$ (11.72g) were dissolved in 400ml of double-distilled H_2O. The solution was adjusted to pH9.6.

2.4.1.7. Phosphate-citrate Buffer (PCB)

Na_2HPO_4 (27.5ml) and 0.1M anhydrous citric acid (24.3ml) were added together and made up to 100ml using double-distilled H_2O, with the pH adjusted to 5.

2.5. Preparation and Purification of Cell Populations

All cell isolation techniques were based on previously optimised systems from within the Anderton group.

2.5.1.1. Isolation of mononuclear cells from CNS tissues

Mice were sacrificed by CO_2 asphyxiation, the aorta was then cut and mice were then perfused with 25ml of PBS through the left ventricle of the heart. Brains were removed by dissection after decapitation. Spinal cords were removed by intrathecal hydrostatic pressure using PBS. The tissues were broken down by mechanical disaggregation and digested, for 30 minutes at 37°C, in wash buffer containing 2.5mg/ml collagenase (Worthington Biochemicals, NJ) and 1mg/ml deoxyribonuclease (Sigma). A single cell suspension was then obtained by further mechanical disaggregation; cells were then washed with RPMI-5.

Cells were then suspended in 30% Percoll (Gibco – made up with RPMI-5) overlaid on 70% Percoll. This allowed the separation of mononuclear cells from the rest of the tissue components, mainly fats, at the gradient interface. Cells were subsequently washed in RPMI-5.

2.5.1.2. $CD4^+$ Cell Purification

Peripheral lymph nodes and spleens were taken from donor mice and single cell suspensions were obtained by mechanical disaggregation. Red blood cells (RBC) were removed after incubation with RBC lysis buffer (Sigma). Cells were then

resuspended in MACS buffer and counted. Cells were incubated with 45µl of MACS buffer and 5µl of MACS anti-$CD4^+$ beads per 10^7 cells for 15 minutes. Cells were then washed in MACS buffer and passed through MS or LS columns according to the manufacturer's instructions (Miltenyi Biotec). Cells retained in the magnetic column ($CD4^+$ fraction) were collected. Samples were stained with anti-CD4-APC and the purity was analysed. Purity obtained was consistently above 90%. Cells were resuspended in PBS and filtered to remove clumps. They were injected intravenously into pre-heated tail veins at a final volume of 200µl per mouse.

2.6. Generation of short-term T cell cultures

Short term cultures, for the investigation of adaptive tolerance, used Tg4 T cells and culture conditions were based on passive transfer culture conditions established by O'Connor et al [268]. Spleen and lymph nodes from naïve Tg4 mice were mechanically disaggregated to produce a single cell suspension. Cells were plated at $5x10^6$ cells per well in RPMI-5 (24-well plate; 1.5ml per well); peptide was added to these cultures to stimulate the cells (10µg/ml of 4Lys, 1.0µg/ml 4Tyr, 0.1 µg/ml 4Tyr and 0.01 µg/ml 4Tyr). Cultures were maintained using a seven day re-stimulation cycle. In the first round of stimulation the Tg4 APC recovered with the T cells were used for stimulation and total cells were plated at $5x10^6$ cells per well in a 24-well plate. In secondary and tertiary rounds of stimulation γ-irradiated (using a caesium isotope, ^{127}Cs) B10.PL splenocytes were used as APC; $1x10^6$ T cells were plated with $3x10^6$ irradiated splenocytes. Cells were stimulated with peptide for approximately 3 days. T cell blasts were then isolated using a Nycoprep 1.077 animal density gradient (Nycomed Pharma, Oslo, Norway - no longer sold) and resuspended in RPMI-5 supplemented with 2% rat concanavalin A supernatant. Cells were rested in this medium for four days before being re-stimulated as above. Flow cytometric analysis and proliferation assays were carried out on cells on day 6 (the day prior to re-stimulation).

2.7. Induction of EAE

2.7.1.1. Single immunisation EAE induction

MOG_{35-55} or 4Lys were administered, sub-cutaneously, in both hind legs with a total of 100µg of peptide, per mouse, emulsified in CFA. 200ng of PTx in 0.5ml PBS was given intraperitoneally on the same day and again two days later.

The $PLP_{139-151}$ peptide was administered into B10.PLxSJL mice sub-cutaneously at 200µg of peptide, per mouse, emulsified in CFA. 200ng of PTx in 0.5ml PBS was given intraperitoneally on the same day and again two days later.

2.7.1.2. Dual immunisation EAE induction

4Lys or 4Tyr (100µg) were administered, sub-cutaneously, in the left leg with 50µg of peptide, per mouse, emulsified in 50µl IFA supplemented with 60µg CpG. After 28 days, EAE was induced using either 100µg 4Lys or 100µg 4Tyr with CFA administered into the right leg. PTx (200ng in 0.5ml PBS) was given intraperitoneally on the same day and again two days later.

2.7.1.3. Passive transfer of EAE

Passive transfer cell culture conditions and timings were based on previous work in the MOG_{35-55} / C57BL/6 model of EAE by O'Connor *et al* [268]. In C57Bl/6 x B10.PL mice, passive EAE was induced by immunising donor C57BL/6 x B10.PL mice with 100µg of MOG_{35-55}, per mouse, emulsified in CFA. Inguinal and paraaortic lymph nodes were removed from these animals ten days later and a single cell suspension was produced as described above. Cells were plated out at $5x10^6$ cells per well in a 24-well plate, with RPMI-5 containing 10µg/ml of MOG_{35-55}, 0.5ng/ml IL-2, 25ng/ml IL-12 and 25ng/ml IL-18 (total well volume of 2ml). After 48 hours, cells were re-stimulated with 2.5ng/ml IL-2 in fresh RPMI-5 added in a 1:1 ratio with the culture volume. Twenty-four hours later T cell blasts were isolated

using Nycoprep 1.077 and then washed with RPMI-5. Cells were filtered to remove clumps and resuspended in PBS at $20x10^6$/ml and then injected intravenously into pre-heated tail veins at a final volume of 200µl ($4x10^6$ T cell blasts per mouse). Purity of cells was not assessed prior to transfer.

Tg4 cells were treated in the same manner as this, except mice were not immunised and both splenocytes and lymph nodes were taken for culture. Varying doses of either 4Lys or 4Tyr were used for stimulation with polarising cytokines as above. In experiments where two rounds of *in vitro* stimulation were required, cells were rested in RPMI-5 supplemented with 2% rat concanavalin A supernatant for four days after primary stimulation. They were then stimulated again for three days, in the absence of polarising cytokines, with irradiated B10.PL splenocytes. Cells were then resuspended in PBS at $25x10^6$/ml and injected intravenously into pre-heated tail veins at a final volume of 200µl ($5x10^6$ T cell blasts per mouse). Mice were given PTx (200ng in 0.5ml PBS) intraperitoneally on the same day and again two days later.

2.7.1.4. Assessment of EAE

Clinical signs of EAE were assessed daily using a standardised scoring index: 0, no signs; 1, flaccid tail; 2, impaired gait and/or impaired righting reflex; 3, partial hind leg paralysis; 4, total hind leg paralysis; 5, hind and fore leg paralysis; 6, moribund or dead. Mice were weighed prior to immunisation and during disease course; mice showing a 20% reduction in weight or a score of 5 two days in a row were culled. Differences in total disease burden between groups were determined using the Mann-Whitney U test.

2.8. Induction of tolerance with soluble peptides

Mice received a single intravenous dose of 200µg of either 4Tyr or $PLP_{139-151}$ in 200µl of PBS seven days prior to immunisation with $PLP_{139-151}$ or 4Lys, respectively, with CFA. This protocol was based on previous work by Anderton *et al* [259].

2.9. Flow Cytometric Analysis

Single cell suspensions were prepared as described above and were resuspended in FACS buffer. Antibodies used for staining were prepared in FACS buffer (clones and concentrations as described in Table 2.1). Cells were incubated with antibody for 15-20 minutes at 4°C before being resuspended in FACS buffer and passed through a Becton Dickinson FACSCalibur or LSR II (BD, Franklin Lakes, NJ, USA). Data obtained was analysed using the FlowJo programme (Treestar, USA).

2.9.1.1. Intracellular staining

For analysis of antigen specific cytokine production, cells were incubated overnight at $6x10^6$ cells per well, in a 24 well plate with 10µg/ml of the relevant peptide. Then they were incubated with 1µl/ml of golgistop (BD Pharmingen, La Jolla, USA) for 4 hours at 37°C. Cells were then stained for surface molecules for 30 minutes at 4°C before being treated with a cytofix / cytoperm kit (BD Biosciences) as per manufacturer's instructions. Cells were then stained for cytokine production for 30 minutes at 4°C before being processed through the LSR II.

Cells analysed for Foxp3 were treated in a similar fashion. The cytofix / permeabilisation kit in this case was from ebiosciences. Again cells were treated as per manufacturer's instructions.

Table 2.1: Targets and Antibody clones used for flow cytometric analysis

Target	Clone	Fluorescent Conjugate	Concentration Used
CD4	RM4.5	FITC/APC/PCP	1/400
CD5	53-7.3	PE	1/200
CD8	53-6.7	PE	1/200
CD25	PC61	PE/APC	1/200
CD44	IM7	PE	1/200
CD69	H1.2F3	Biotin	1/200
CD45.1	A20	FITC/PE	1/200
CD90.1	HIS51	FITC/APC	1/200
TCR-β*	H57-597	APC	1/200
Foxp3*	FJK-16s	FITC/APC	1/200
IL-2	JES6-5H4	PE	1/200
IFN-γ	XMG1.2	FITC	1/200
Fc Receptors	2.4G2	unconjugated	1/50

* From ebioscience. All other antibodies from BD Pharmingen.

2.10. *In vitro* assessment of T cell function

2.10.1.1. Recall response proliferation assays

Single cell suspensions were made from lymph nodes and splenocytes. Cells were cultured, in triplicate, using 96-well flat bottomed plates (at $6x10^6$ cells/ml per well) against varying doses of peptide. After 48 hours, 0.5μCi/well of tritiated thymidine (^{3}H-dThd, Amersham) was added to the cultures. After an overnight incubation the cultures were harvested and the thymidine incorporation was measured using a liquid scintillation β-counter (Wallac/Perkin-Elmer, Waltham, USA).

2.10.1.2. Cytokine production ELISA

Lymph node cells and splenocytes were mechanically disaggregated to produce single cell suspensions which were then cultured, in 96-well flat bottomed plates, with varying doses of peptide as indicated on each graph. Plates were incubated at 37°C for 48 hours before analysis.

Anti-IL-2 or anti-IFN-γ capture antibodies (clones JES6-1A12 and R4-6A2 respectively, from BD Pharmingen) were diluted to 2µg/ml and 1µg/ml respectively in bicarbonate buffer and were used to coat 96-well MaxiSorb microtiter plates (Nunc International, Roskilde, Denmark), at 50µl per well. Plates were incubated at 4°C overnight. After being washed twice in PBS/0.1%Tween (20) the plates were blocked with PBS/1%BSA (200µl per well) for 1 hour at 37°C. The plates were then washed again, twice with PBS/0.1%Tween (20) and twice with PBS alone. IL-2 and IFN-γ cytokine standards were then added in duplicates (100µl per well), at top concentrations of 1000pg/ml and 100ng/ml respectively, in two-fold dilutions to produce a 10-point standard curve. Duplicate samples of culture supernatants (100µl per well) were added to wells in the MaxiSorb plate and 4 blank wells with 100µl PBS/1%BSA were also prepared.

Plates were then incubated at room temperature for 2 hours before washing four times with PBS/0.1%Tween (20). A secondary, biotinylated anti-cytokine detecting, antibody (anti-IL-2-capture, clone JES6-5H4 and anti-IFN-γ-capture, XMG1.2; BD Pharmingen) diluted in PBS/1%BSA was added at this stage (100µl per well, at a final concentration of 0.5µg/ml) and the plates were incubated at room temperature for 1 hour. This was followed by 6 washes with PBS/1%BSA prior to incubation with 100µl per well of ExtrAvidin peroxidase (made up to final concentration > 2µg/ml in PBS/1%BSA; Sigma) at room temperature for 30 minutes. Plates were again washed 6 times with PBS/1%BSA. Tetramethylbenzidine (TMB; Sigma) solution (prepared by adding 100µl of TMB in DMSO to 9.9ml of PCB and 3µl of hydrogen peroxide) was added to the plates (100µl per well) and was followed by

100ml per well of 2M sulphuric acid to stop the reaction. Absorbance values were read at 450nm using a Multiskan plate reader (Labsystems, Basingstoke, UK).

2.11. Statistics

The Mann-Whitney test was performed for statistical analysis of EAE disease. This non-parametric rank sum test was chosen because the measurements of EAE scores are discrete. Previous reports from the Anderton group have also made use of the Mann-Whitney test and the results have been used and accepted [99; 260].

In statistical analysis of cell surface ligand expression and analysis of proliferation assay data, an unpaired student's t test was performed. This parametric test was used as these data were considered indiscrete variables.

Chapter 3:

Testing disease models based on both MOG_{35-55} and MBP_{Ac1-9} to study epitope spreading

3. Testing disease models based on both MOG_{35-55} and MBP_{Ac1-9} to study epitope spreading.

3.1. Introduction

Previous work by the Anderton group made use of the C57BL/6 x B10.PL mouse [174]. This mouse has both I-A^u and I-A^b MHC haplotype. A striking feature of this F_1 mouse, which sets it apart from its parental strains, is that no disease was observed despite the introduction of either MOG_{35-55} or 4Lys within a standard EAE-inducing immunisation. In fact disease was only observed with 4Lys if there was an adoptive transfer of Tg4 cells prior to the immunisation. This limitation of disease to the introduction of 4Lys relevant cells is advantageous in this mouse because it enables the tracking of the disease relevant cell population, using congenic markers such as CD90.1 or CD45.1 which are not present in the host animals.

One of the primary aims of the experiments detailed in this chapter was to establish a relapsing-remitting form of EAE using both the MOG_{35-55} and MBP_{Ac1-9} peptides to allow the observation of intermolecular epitope spreading, which has been suggested as the causative mechanism for RR-EAE. Intramolecular epitope spreading, with MBP_{Ac1-9}, was previously noted in B10.PL x SJL mice [265] and both intra- and intermolecular spreading, caused by $PLP_{139-151}$, was noted in SJL mice [267].

A second goal was to establish EAE in C57BL/6 x B10.PL mice without the use of transgenic cells. This would provide the opportunity to examine EAE with a diverse repertoire of T cells expressing different TCR. This system could then be exploited to enable the examination of the effects of molecular mimicry in EAE; by using a superagonist APL to mimic a strong stimulus given by a foreign antigen and create an antigen experienced cohort of cells which could be challenged at a later time point with the wild-type native epitope that shares sequence homology with the superagonist.

This model could be used to clarify the mechanisms of peripheral tolerance by allowing the examination of the fate of a 4Lys experienced T cell repertoire after a challenge with superagonist peptide. Is the immune system able to maintain the repertoire and avoid deletion of cells bearing TCR that may be required at a later time point to fight a different immunological challenge? If so then how is this achieved?

3.2. Developing a model to study the contribution of MOG or MBP peptides to epitope spreading

In an attempt to develop a model to observe epitope spreading using MOG_{35-55} and 4Lys, transgenic cells bearing TCR relevant to these peptides (2D2 [43] and Tg4 [49] cells respectively) were used in concert within the C57BL/6 x B10.PL mouse. $1.5x10^6$ 2D2 $CD90.1^+CD4^+$ and 10^6 Tg4 $CD45.1^+CD4^+$ cells were transferred together into C57BL/6 x B10.PL mice. These mice were subsequently immunised with either the MOG_{35-55} or MBP 4Lys peptides (Fig. 3.1A).

Only 50% of 4Lys immunised mice showed signs of EAE with 25% of the total group reaching a fatal clinical score; the remaining mice showed low levels of disease. Clinical signs of EAE were observed in 75% of the MOG_{35-55} immunised mice (Fig. 3.1B). A small number of mice immunised with MOG_{35-55} relapsed with mild disease (mean score 1) around day 30.

Lymph nodes, spleen and CNS were taken from sick mice at day 8 of disease; cells were stained for flow cytometric analysis to enable tracking and to observe phenotypic changes. These mice were excluded from the time course analysis because scoring for clinical signs of disease in this experiment only began at day 7 (Fig 3.1B). Despite the observation of disease in both immunisation groups the flow cytometric analysis showed that no 2D2 cells could be tracked within the C57BL/6 x B10.PL mouse. Tg4 cells were however not only present in both immunisation groups but were also seen to be enriched within the CNS; Tg4 cells were also present in the lymph nodes and spleen of the non-immunisation control mice (Fig. 3.2).

3.3. MOG_{35-55} relevant TCR transgenic cells fail to survive adoptive transfer into C57BL/6 x B10.PL mice

The lack of visibility of the 2D2 cells in either immunisation group suggested that these cells were not surviving transfer into the C57BL/6 x B10.PL mouse.

Experiments were designed to compare the viability and cell survival of the 2D2 $CD4^+$ cells after adoptive transfer into the C57BL/6 x B10.PL and the C57BL/6 mouse.

The adoptive transfer of $0.5x10^6$ 2D2 $CD90.1^+CD4^+$ cells into either C57BL/6 or C57BL/6 x B10.PL mice was followed, after 24 hours, by immunisation with MOG_{35-55} (Fig. 3.3A); tissue samples were analysed prior to immunisation. Even at this time point (day 1), analysis of the lymph nodes of the C57BL/6 x B10.PL mice showed a 58% reduction in the frequency of 2D2 cells compared to those of the C57BL/6 mice. This difference fell to 37% in the spleen (Fig. 3.3B). At day 5 post-cell transfer (4 days post-immunisation), 2D2 cells in the C57BL/6 mice showed an expansion in numbers in both lymph nodes and spleen whereas the C57BL/6 x B10.PL mice showed no expansion; with the frequency of 2D2 cell remaining similar to day 1 figures (Fig. 3.4). This was consistent with day 8 data where C57BL/6 x B10.PL mice showed a 99.5% and 93.75% reduction in 2D2 cell frequency in the lymph node and spleen respectively compared to C57BL/6 mice.

3.4. C57BL/6 x B10.PL APC are capable of stimulating 2D2 $CD4^+$ cells *in vitro*

In order to assess if the poor survival of 2D2 $CD4^+$ cells was due to a lack of *in vivo* stimulation in the C57BL/6 x B10.PL mice, 2D2 proliferation assays were carried out using irradiated and non-irradiated APC from both the C57BL/6 x B10.PL and the C57BL/6 mice. Lymph nodes and spleen were taken from 2D2 mice and were positively selected for CD11c; the negative fraction from this selection was then positively selected for $CD4^+$ using MACS. $4x10^4$ 2D2 $CD4^+$ cells were added to 10^5 C57BL/6 x B10.PL or C57BL/6 irradiated or non-irradiated APC (Fig.3.5A). Irradiated APC groups were included to show that proliferation was not a result of expansion of the C57BL/6 x B10.PL or C57BL/6 cell populations. After 30 hours cells were pulsed with ^3H (thymidine), the incorporation of this was used as readout of proliferation and was measured as counts per minute (cpm) at 48 hours (Fig.3.5B).

Although data show a 50% reduction in proliferation of 2D2 cells with APC from C57BL/6 x B10.PL compared to those from C57BL/6 (at 5µg/ml of peptide) there is no significant difference observed between the proliferative potential of 2D2 cells with either irradiated or non-irradiated APC from each strain (unpaired t test). The data show that proliferation occurs when antigen is presented to 2D2 cells by C57BL/6 x B10.PL *in vitro* indicating that 2D2 cells have the potential to be stimulated in C57BL/6 x B10.PL mice. Increased proliferation with non-irradiated APC can be explained by the active MOG_{35-55} relevant host repertoire of $CD4^+$ cells that exists in the C57BL/6 mouse.

3.5. Developing a passive transfer model for the C57BL/6 x B10.PL model

Due to the poor viability of 2D2 cells within the C57BL/6 x B10.PL mouse, the behaviour of a MOG_{35-55} relevant repertoire of T cells had to be analysed via a different protocol. A previously published passive transfer protocol, from O'Connor *et al*, was modified for using the C57BL/6 x B10.PL mice as the source of both cells - donor and host [268]. C57BL/6 x B10.PL mice were immunised with 100µg of MOG_{35-55} in CFA and were then left for 10 days before inguinal and para-aortic lymph nodes were removed. The cells from these nodes were cultured for 72 hours with MOG_{35-55}, IL-2, IL-12 and IL-18 to polarise the $CD4^+$ cells into Th_1 type $CD4^+$ cells. At day 3 the cells were transferred into naïve F_1 mice followed by two shots of PTx, one on the same day and another 2 days later (Fig 3.6A).

Immunisation of mice with peptide and CFA leaves a long-term depot of antigen for cells to be continually activated from. This can cause disease even after a primary course of T cell activation and therefore potentially after disease recovery has occurred; passive transfer models eliminate this problem.

The data show that the *in vitro* polarised cells do cause disease in the C57BL/6 x B10.PL mice; this disease was monitored for 14 days. Clinical signs of EAE first

appear at day 5 (2 days earlier than that seen in the traditional actively induced model of EAE - peptide + CFA + PTx), with the peak of disease at day 10. The maximum clinical score seen in any one mouse was grade 3 and the mean maximum clinical score was 2.25 (Fig 3.6B).

The *in vitro* stimulation was repeated with naïve Tg4 cells; $5x10^6$ blasting cells were transferred into C57BL/6 x B10.PL alongside two doses of PTx (Fig 3.7A). These *in vitro* stimulated Tg4 cells were highly pathogenic. Disease course shown is a compilation of two different experiments; once again, disease began at an early time-point (relative to an active induction of EAE - at day 5 rather than at day 7) however in this passive model disease lasts for at least 20 days (Fig 3.7B). The maximum clinical score seen in any one mouse, receiving stimulated Tg4 cells, was 5 with the maximum mean group clinical score of 3.5. Mice that were given 'unstimulated' (naïve) Tg4 cells did not present with any clinical score throughout the 25 day period. Mice from both passive and naïve transfer were culled at day 16 and spleen and CNS were processed for flow cytometric analysis. Data show that out of all $CD4^+$ cells in the spleen the mean number of Tg4 cells was 8.59%; this number increased dramatically to 57% of total $CD4^+$ cells when CNS was examined (Fig. 3.8A). These observations were repeated at day 25 when the experiment was terminated due to excessive weight loss in the mice(Fig. 3.8B).

Further experiments attempted to track *in vitro* stimulated 2D2 cells within the C57BL/6 x B10.PL on the assumption that *in vitro* stimulated (and therefore previously activated) cells would have an advantage in terms of viability and pathogenicity to naïve adoptively transferred 2D2 cells. However, as in previous data from the adoptive transfer of 2D2 into the C57BL/6 x B10.PL model, very few of these cells survived the transfer when compared to transfer into the C57BL/6 mouse (Fig 3.9).

In an attempt to prevent any possible rejection of the 2D2 cells from the C57BL/6 x B10.PL system, an F_1 mouse was produced by crossing 2D2 mice with B10.PL mice.

Cells from these F_1 mice were passively transferred into the C57BL/6 x B10.PL mice, once again the 2D2 cells showed poor viability as shown by Figure 3.10.

3.6. Developing a non-transgenic model of EAE in C57BL/6 x B10.PL

As mentioned earlier, C57BL/6 x B10.PL mice do not show clinical signs of EAE after a single immunisation alone, with either MOG_{35-55} or 4Lys, and indeed that was the previous dogma. Experiments in our laboratory have recently described the induction of EAE using a double-immunisation protocol using MOG_{35-55} peptide in C57BL/6 mice [269]. Peptide is introduced with 60µg CpG and IFA into one hind leg and is followed 28 days later with peptide and CFA into the other followed by the intraperitoneal administration of PTx to induce EAE.

The peptide used here was 4Lys (Fig. 3.11A). 75% of the mice showed clinical signs of EAE, with a mean peak clinical score of 2.75 (Fig. 3.11B) with a mean duration of 8.5 days. The proliferation of splenocytes from these mice was normal when tested against 4Lys *in vitro* (Fig. 3.11C). These data show that a double-immunisation protocol can allow us to bypass the use of transgenic cells in the C57BL/6 x B10.PL mice.

3.7. Discussion

The experiments outlined in this chapter represent attempts to establish RR-EAE models of EAE with the MOG_{35-55} and MBP_{Ac1-9} peptides which were unsuccessful. Disease was so severe in 25% of mice immunised with 4Lys that these mice showed no recovery for the duration of the experimental time course; those immunised with MOG_{35-55} became ill, showed good recovery and a small number relapsed with mild disease (mean score 1) around day 30 (Fig. 3.1). Epitope spreading is more clearly indicated by data showing that the immunisation of C57BL/6 x B10.PL mice with MOG_{35-55} allowed the entry and expansion of MBP_{Ac1-9} relevant cells in the CNS (Fig. 3.2); suggesting that MBP was released from the CNS and allowed the homing of the relevant cells. This would have been more clearly defined had analysis of CD44 expression (a marker of antigen experienced cells) been investigated.

Other data exist showing that epitopes of MOG, specifically MOG_{92-106}, can be used within bystander activation models in SJL mice to induce relapses against secondary myelin epitopes which are accompanied with extensive B cell reactivity [270]. Future experiments examining the potential of MBP and MOG related epitope spreading would benefit from the analysis of antibody class switching; class switching would indicate a shift in B cell reactivity and therefore a switch in the repertoire of T cells mediating disease.

The data presented in this chapter show the strong viability of the Tg4 cells after transfer into other histocompatible ($H\text{-}2^u$) strains. In each transfer of these cells, disease was manifested and cells could be tracked and found to be enriched in the CNS. None of the experimental data presented here show that 2D2 cells can match the viability, traceability and proliferative potential of the Tg4 system during disease, if in fact disease is manifested at all.

The reduced ability to induce disease, shown in Figure 3.1, cannot be accounted for by the suggestion that there is a problem with antigen presentation by C57BL/6 x B10.PL APC to 2D2 $CD4^+$ cells. *In vitro* assays comparing the proliferative

potential of 2D2 cells against APC from C57BL/6 mice or C57BL/6 x B10.PL mice show that although the potential is lowered in the latter strain, it is still strong.

This is further supported by the successful development of the passive transfer model using MOG_{35-55} within C57BL/6 x B10.PL mice where a MOG_{35-55} immunisation is used to produce an antigen experienced cohort of cells within F_1 mice, which when stimulated *in vitro* and then transferred can induce disease in the same mice.

The reasons for the poor survival of the 2D2 cells are unclear. They were bred onto a C57BL/6 background and therefore should have the capacity to survive C57BL/6 x B10.PL hosts. Transfers of the 2D2 cells into C57BL/6 mice have not been seen to be successful in inducing disease although flow cytometric analysis from the Anderton group has been able to track their movement into the CNS (M. Leach, personal communication); however doubts remain as to whether their migration to the CNS is antigen specific. Another feasible option is that the 2D2 cells are being killed off by NK cells within the C57BL/6 x B10.PL mice. NK cells have inhibitory receptors that scan cells for low levels of MHC; cells such as these can be killed off via perforin and granzymes.

Although Paterson [29], is credited with the initial passive transfer of EAE with lymph node cells, the technique most commonly used today is based on work, in SJL mice, by Pettinelli *et al* in the 1980s [271]. The passive transfer model serves as a powerful tool to examine the progression of disease. This method of EAE induction separates the induction phase (immunisation) from the effector phase (disease progression) by confining each phase to separate groups of animals. The model's advantages include the elimination of the presence of non-disease relevant antigens (from *Mycobacterium tuberculosis* in CFA) and may be considered 'cleaner' because the induction of disease occurs through the transfer of only the disease inducing cells, in the absence of antibody and antigen. It also allows for more direct tracking of the disease relevant cells.

Disadvantages include the requirement for an *in vitro* tissue culture stage and the increased numbers of mice required as well as the large amounts of peptide needed for both immunisation and *in vitro* challenge, relative to active induction alone. Also there is a greater temporal cost, the induction phase in host mice takes 10 days followed by a further 3 days *in vitro* before the cells can be transferred. In the data shown here the time for a clinical score to be observed after immunisation in passive induction is 18 days compared to 7 days in active induction.

The development of the double-immunisation protocol within the 4Lys / C57BL/6 x B10.PL model is important in allowing the progression of disease without the requirement for transgenic cells seen previously [269]. This system can be used in allowing the examination of the induction of tolerance in an antigen experienced cohort of host cells and combined with the MBP_{Ac1-9} APL system allows the investigation of molecular mimicry within C57BL/6 x B10.PL mice.

This chapter focuses on attempts to define a RR-EAE model using the MBP_{Ac1-9} and MOG_{35-55} epitopes which were unsuccessful due to the poor viability of the 2D2 transgenic cells. The chapter describes the successful modelling of passive disease using MOG_{35-55} to prime host cells *in vivo* and *in vitro.* The Tg4 cell passive transfer model is also defined as well as a dual immunisation protocol, both of which were used as base models for further investigations into adaptive tolerance.

Chapter 4:

Developing a model to examine therapeutic immune tolerance in epitope spreading

4. Developing a model to examine therapeutic immune tolerance in epitope spreading.

4.1. Introduction:

The progression of RR-MS and RR-EAE is a cyclical process created by the positive and negative feedback nature of the immune system. Immunological cycles depend on the mechanisms which control clonal expansion and death of disease relevant cells. The phenomenon of epitope spreading has long been implicated within the relapsing-remitting course of multiple sclerosis and its animal model, EAE [265, 267]. The destruction of the myelin sheath, due to the focus of the immune response on an initial dominant epitope, leads to the release of other cryptic epitopes against which different pathogenic T cells can react. Consequently the focus of the immune response deviates and relapses are seen. One of the few positive aspects of epitope spreading may be the potential that antigen release may allow the progression of bystander suppression [272].

Anderton and Wraith [259] first addressed the question of whether the MBP_{Ac1-9} APL, 4Tyr [50, 256] could suppress disease induced with the wild-type I-A^u-restricted 4Lys or with the I-A^s-restricted epitope $PLP_{139-151}$. Evidence from experiments involving the intra-nasal administration of 4Tyr, prior to EAE induction with whole myelin, showed that the APL treatment was able to suppress disease induced with 4Lys and MBP_{89-101} (intra-molecular suppression) but was ineffective at suppressing disease with $PLP_{139-151}$ (inter-molecular suppression).

The situation was reversed when the I-A^s-restricted $PLP_{139-151}$ was administered prior to EAE induction with either whole myelin or with MBP derived epitopes; disease was suppressed and T cells showed reduced proliferative potential against all three peptides. Worthy of note is the fact that lymph node responses to MBP were only suppressed by the $PLP_{139-151}$ treatment when EAE was induced by whole myelin rather than the individual peptides. The use of whole myelin, containing many more epitopes than the three used initially, shows that $PLP_{139-151}$ has a wide-ranging

capacity to suppress disease. Anderton *et al* concluded that the requirement for whole myelin suggested a need for co-presentation of $PLP_{139-151}$ at the same time and site for suppression to occur.

Although a switch from a Th_1 to Th_2 cytokine milieu was not supported by the data from that previous study, the disparity in the 'bystander suppression' may be explained by the precursor frequency of T cells reactive to the MBP or $PLP_{139-151}$ epitopes. Whereas $PLP_{139-151}$ is conserved within the CNS, MBP is known to be a component of the sheath in peripheral nerves [273] and data show that it may be presented in the thymus of a young mouse [274] – both these factors would help to reduce the frequency of MBP-reactive cells. We know that there is a much higher frequency of $PLP_{139-151}$ relevant T cells in the periphery, as they are able to escape from thymic selection [236]. This failure is due to the expression of DM20 in the thymus, a spliced variant of PLP with a deletion at residues 116-150 [275].

This lower initial frequency of cells reactive to MBP means that fewer regulatory cells may be induced by the 4Tyr peptide treatment in comparison to the $PLP_{139-151}$ treatment. This may in turn help to explain the less potent inter-molecular suppressive effect of the 4Tyr.

Lower numbers of 4Lys reactive cells can be compensated for with the introduction of MBP_{Ac1-9} relevant Tg4 transgenic cells [49] and in this chapter Tg4 cells were used in combination with the $H\text{-}2^{u,s}$ B10.PL x SJL mouse to develop a model of EAE. This model has been used to evaluate the potential of tolerised transgenic cells to limit the severity of disease induced with a different myelin auto-antigen. Previous studies from the Anderton group have shown a relapsing-remitting form of disease in B10.PL x SJL mice immunised with the 4Lys epitope [276].

The work discussed below focuses on disease induced with the $PLP_{139-151}$ epitope in B10.PL x SJL mice and the findings therein that the transfer of Tg4 cells enhances single phase disease to a relapsing-remitting state. Furthermore the question of

whether the tolerisation of the Tg4 cell population could ameliorate disease induced by either the $PLP_{139-151}$ or the 4Lys epitope within the F_1 mice was addressed.

4.2. The transfer of Tg4 transgenic cells causes a relapsing-remitting in B10.PL x SJL mice

Tg4 cells, purified using MACS positive bead selection for CD4, were adoptively transferred into B10.PL x SJL (H-$2^{u,s}$) mice in order to investigate the possibility that disease induced by $PLP_{139-151}$ could diverge to the Tg4 relevant 4Lys epitope, after an initial phase of disease. The B10.PL x SJL mouse is $CD45.1^{+}CD45.2^{+}$ and therefore in later experiments Tg4 cells, lacking CD45.1 could be tracked using flow cytometry based on the lack of staining with anti-CD45.1.

Either $0.5x10^6$ or $1x10^6$ Tg4 cells were transferred into B10.PLxSJL mice which were subsequently immunised with 200µg of $PLP_{139-151}$ (Fig 4.1A). Two control groups were also set up, the first was immunised with $PLP_{139-151}$ but was not given the Tg4 cell transfer and the second was given a transfer ($1x10^6$ Tg4) but no immunisation. The mean clinical score was monitored for a period of 55 days following immunisation to attempt to track further relapses however no disease was observed beyond day 35 (Fig 4.1B).

Disease course analysis showed that only mice that received both Tg4 transfer and $PLP_{139-151}$ immunisation showed a relapsing-remitting course of disease. Those that received only immunisation showed a single phase of disease with no relapse. This alone suggested that the Tg4 cells that had been transferred were in some way responsible for the relapses seen. The first phase of disease was not significantly different between groups where either $0.5x10^6$ or $1x10^6$ Tg4 cells were transferred despite the earlier recovery observed in mice given $0.5x10^6$ cells (Fig 4.1; Mann-Whitney Test; $p = 0.2949$). Mice that received immunisation alone also showed a non-significant difference compared to those receiving $1x10^6$ Tg4 cells (Fig 4.1; Mann-Whitney Test; $p = 0.3337$).

Repeat experiments, this time using 4Lys immunisations, as positive controls for RR-EAE, showed similar disease patterns as in Figure 4.1. $PLP_{139-151}$ immunised mice were scored over a period of 45 days. During the primary phase of disease, starting at

around day 10 and finishing around day 25 there was no significant difference between the immunised groups which received Tg4 cells and those that did not (Fig 4.2; Mann Whitney Test; p = 0.1576). It was the period of time between day 25 and day 35 that showed significant difference in total disease course (Mann Whitney Test; p = 0.0170). Relapses in disease were only observed in groups where Tg4 cells had previously been transferred (Fig 4.2). The 4Lys control immunisation result in the rapid death of the immunised animals (within a period of 15 days) so the entire disease course could not be monitored for full comparison to the $PLP_{139-151}$ immunisations. These data suggest that Tg4 cells are having an impact on the course of disease in this $PLP_{139-151}$ / B10.PLxSJL model of EAE.

4.3. Epitope spreading to the 4Lys epitope is not observed in $PLP_{139-151}$ induced disease

To determine the likelihood of Tg4 cells having an active effect on the course of disease, as suggested above, Tg4 cells were adoptively transferred into B10.PL x SJL mice which were subsequently immunised with either 4Lys or $PLP_{139-151}$. Lymphoid organs and the CNS were analysed by flow cytometry on day 14, with the proliferative potential of splenocytes being tested *in vitro*.

Figures 4.3A and 4.4 show that at peak of disease where one would predict maximum inflammatory infiltrate, day 14, there was a significant enrichment of Tg4 cells within the CNS of 4Lys immunised mice when compared to the peripheral lymphoid organs. Mice immunised with $PLP_{139-151}$ however showed no enrichment in the CNS.

Spleens were taken from animals after recovery from relapse (day 35 - Fig 4.5) and cells were challenged *in vitro* with varying concentrations of 4Lys or $PLP_{139-151}$. After 72 hours cultures were pulsed with ^{3}H (thymidine), the incorporation of this was used as readout of proliferation and was measured as counts per minute (cpm). Responses against $PLP_{139-151}$ were similar in all groups, even where no Tg4 cells had

been transferred confirming that these cells have no impact on the host immune response against the $PLP_{139-151}$ peptide. No significant responses were seen when splenocytes were challenged with the 4Lys peptide, even at high concentrations (Fig 4.5B).

4.4. Administration of soluble 4Tyr ameliorates disease in the Tg4 / B10xSJL model

In an attempt to manipulate the Tg4 cells in this model and in turn manipulate the effect these cells would have on the course of disease, $1x10^6$ Tg4 cells were transferred into B10.PLxSJL mice and then 4Tyr in PBS was administered to attempt to tolerise the cells *in vivo* seven days prior to a $PLP_{139-151}$ immunisation (Fig 4.6A), the protocol being based on previous work by Anderton *et al* [259]. In a separate group of mice $PLP_{139-151}$ was used with PBS in a tolerising protocol in EAE induced by 4Lys (Fig 4.7A). These mice received Tg4 cells at a later timepoint to those treated with 4Tyr because they would not have had stimulus to survive *in vivo* in the absence of a relevant antigen. Mice were scored for disease for a period of 40 days after immunisation.

Mice that were given 4Tyr after the Tg4 transfer showed a significantly different course of disease ($p<0.0001$; Mann-Whitney Test) to those that received the PBS alone over several experimental time courses. Data from two separate experiments were collated for disease score and is shown in Figure 4.6B. In both groups, clinical signs of disease were observed at day 5 and were of similar severity and duration. The first relapse in the 4Tyr treated group is observed on day 42 (22 days after the non-treated group) with only 2 out of 15 mice relapsing with a mean clinical score of 0.5. Non-4Tyr treated mice had a mean clinical score of 1 during relapse (6 of 12 mice relapsed). None of the mice treated with 4Tyr presented signs of a further relapse, with the caveat being that mice were culled at day 70 and may have relapsed at a later time point that this (Fig 4.8A & B). The data show more than one relapse in the mice that were treated with PBS alone, at days 38 and 51; this was not seen in previous experiments in which disease was only monitored till day 35.

In mice treated with $PLP_{139-151}$ and PBS and subsequently immunised with 4Lys (Fig 4.7A), the initial phase of disease was lower in severity than those mice given only PBS (Fig 4.7B). The severity of the primary relapse was similar between groups and a secondary relapse was only seen in the mice treated with $PLP_{139-151}$, again with the caveat that the disease course was only monitored for a period of 40 days.

4.5. MBP_{Ac1-9} relevant Regulatory T Cells can be seen in the CNS of $PLP_{139-151}$ immunised mice

In order to explain the differences in disease course seen above and to assess the effects the 4Tyr on the transferred Tg4 population of cells we culled mice during the recovery of the primary phase of disease (day 14) and cells from the inguinal and paraortic lymph nodes, spleen and CNS were taken and stained for flow cytometry analysis with Tg4 cells in this transfer model traceable because of their lack of the CD45.1 marker.

In animals not given 4Tyr (prior to cell transfer and immunisation) Tg4 cells did appear to be present in the spleen (Fig 4.9A). However the flow cytometry staining suggested that there were no transgenic cells present in either the lymph nodes or the CNS (Fig 4.9A & Fig 4.10A). Similarly animals treated with 4Tyr showed few Tg4 cells present in the lymph nodes and spleen (Fig 4.9A). However, in contrast to the PBS treated mice, CNS samples from the 4Tyr treated mice showed a distinct population of Tg4 $CD4^+$ cells (Fig 4.10B). The staining showed a 5-fold enrichment of cells within the CNS compared to the spleen sampled (0.99% of total $CD4^+$ cells in the CNS compared to 0.18% in the spleen). Although t test analysis of the data suggests no significant difference, our flow cytometry observations clearly indicate a defined population of Tg4 cells in the CNS of the 4Tyr treated mice (Fig 4.10B) whereas the PBS treated mice (Fig 4.10A) showed no greater degree of staining than the control sample (Fig 4.10C).

Further analysis of this infiltrating population of Tg4 cells showed that a mean of 45% of these cells expressed Foxp3 (Fig 4.9B & Fig 4.11). Analysis of the host populations of both groups of mice showed no significant difference in the size of the host regulatory T cell population (Fig 4.9C: p=0.4548; unpaired t test). To check if cells were unable to migrate to the CNS we obtained flow cytometry data on CD69 and CD49d (VLA-4 – required for cell migration to the CNS) expression within the lymph nodes. We also analysed expression of CD44 in the CNS. None of these analyses revealed any difference in expression between the two groups of mice (data not shown).

Splenocytes from the animals, again taken at day 14, were cultured with either 4Tyr or $PLP_{139-151}$ overnight and subsequently stained to measure the production of either IFN-γ or IL-17. Although no differences were seen between the groups, the *in vitro* challenge with the $PLP_{139-151}$ produced a greater amount of both IFN-γ and IL-17 in both groups of mice (Fig. 4.12).

4.6. Discussion:

Epitope spreading has generally been accepted to be a result of the progression of the immune response from one epitope to another [267] resulting in multi phase disease. Early experiments in this laboratory modelled relapsing-remitting EAE using B10.PL x SJL mice immunised with the MBP epitope 4Lys [276].

Here, MBP_{Ac1-9} relevant Tg4 cells were transferred into animals immunised with $PLP_{139-151}$ in order to examine the possibility of an intermolecular spread of disease (from $PLP_{139-151}$ to 4Lys). The presence of relapses clearly implicate the transferred Tg4 cells in mediating some aspect of disease; whereas the normal $PLP_{139-151}$ immunisation shows no relapse, the introduction of Tg4 cells changes the single phase disease course to a characteristic relapsing-remitting disease course.

Paradoxically, the data showed no evidence of a response to the 4Lys peptide from cells taken from these $PLP_{139-151}$ immunised mice. The Tg4 cells transferred into mice immunised with $PLP_{139-151}$ showed comparatively little migration into the CNS when examined alongside mice immunised with 4Lys. Splenocytes, from mice immunised with $PLP_{139-151}$, showed poor proliferative potential, comparable to negative controls, when challenged *in vitro* with 4Lys.

This may be explained by recent work which attempts to define the location at which epitope spreading occurs. By introducing CFSE labelled $PLP_{139-151}$ relevant transgenic cells into mice immunised with $PLP_{178-191,}$ McMahon *et al* [277] observed that these cells did not proliferate in the peripheral lymphoid compartments but showed extensive proliferation in the CNS. As expected, they did proliferate well in both periphery and CNS of mice immunised with $PLP_{139-151}$. Flow cytometric analysis of activation markers confirmed these data to suggest that epitope spreading occurs primarily in the CNS and not in the periphery. These observations may help to explain why the splenocyte proliferation data shown in this chapter shows no evidence of a 4Lys response.

The previous dogma would predict that the Tg4 cells would have been activated by APC in the peripheral lymphoid compartments and would then be recruited into the CNS. In contrast to this, McMahon's conclusion suggests that naïve Tg4 cells would need to migrate to the CNS during the first phase of $PLP_{139-151}$ induced disease to be presented with 4Lys by APC. This may support some of the data in this chapter in that some migration of Tg4 cells into the CNS in $PLP_{139-151}$ induced disease is observed. The marked increase in Tg4 numbers in the 4Lys immunised mice due to an almost immediate expansion of these cells in the periphery can explain their larger population in the CNS compared to those mice immunised with $PLP_{139-151}$.

It may be feasible that the small number of Tg4 cells that do travel to the CNS mediate enough damage to reveal other disease mediating epitopes. It seems likely that relapses would follow the hierarchal pattern established in SJL mice, firstly directed against $PLP_{178-191}$ and then against MBP_{89-101} [267]. It may also be feasible that the Tg4 cells travelling to the CNS create a cytokine milieu that is effective in activating surrounding cells which in turn mediate disease.

Previous studies have described that soluble peptides are an effective means of limiting the pathogenicity of certain autoimmune diseases including EAE [258], arthritis [278] and diabetes [279]. The mechanisms of tolerance have not fully been identified however. Anderton and Wraith [259] demonstrated the potential of using soluble peptides in conjunction with the MBP_{Ac1-9} APL system in the B10.PLxSJL mouse. They were unable however to show that the 4Tyr APL could suppress disease induced by $PLP_{139-151}$.

The tolerisation of B10.PLxSJL mice with $PLP_{139-151}$ has been repeated here with several changes; the tolerising peptide was administered i.v with PBS rather than i.n. and Tg4 cells were administered. The primary phase of disease is remarkably similar to that seen by Anderton and Wraith. However in a change to their observations, the data in this chapter show relapses in both $PLP_{139-151}$ treated and PBS treated mice can recovery fully from disease between the relapses with the introduction of Tg4 cells.

The experiments shown here overcome the numerical limitations of previous work by artificially increasing the precursor frequency of T cells relevant to 4Lys through the introduction of Tg4 cells which bear the moderate affinity receptor for 4Lys. The data show that 4Tyr treatment in this scenario is not able to limit the severity of the initial phase of disease caused by $PLP_{139-151}$ but does substantially delay the onset of relapse. This in turn supports the notion that the Tg4 cells are relevant to relapses as when they are 'silenced' there is no relapse. Further investigations into the hierarchy of epitopes to which the immune response is directed to cause relapses in disease would address the question of whether the relapse has been delayed or if a relapse directed at a certain epitope is missing in 4Tyr treated mice - hence no disease in the time frame where non-4Tyr treated mice relapse.

The data show the presence of Tg4 $CD4^+$ cells within the CNS. The Foxp3 marker is expressed on 3 to 5% of naïve Tg4 cells pre-transfer (S. Anderton, unpublished observations). However, here 45% of the Tg4 cells in the CNS expressed Foxp3. This suggests that either the Tg4 Tregs have been recruited to the CNS and expanded or that Tg4 $CD4^+$ cells have migrated to the CNS and been converted into Tregs expressing the Foxp3 marker due to the milieu present in that tissue. Either way, the presence of these Foxp3 cells suggests that they may be responsible for limiting the continued severity of disease.

Chapter 5:

Investigating the Role of Adaptation in Limiting Pathogenicity of Autoimmune Disease

5. Investigating the Role of Adaptation in Limiting Pathogenicity of Autoimmune Disease.

5.1. Introduction

Due to the cross-reactive nature of the TCR, a single T cell has the ability to respond to epitopes from both infectious agents and self tissue [153, 154]. One of the factors suggested to initiate autoimmune disease is molecular mimicry. The molecular mimicry theory suggests that an immune response initially directed at a non-self antigen by a T cell can spread to a self antigen because of the cross-reactivity of the TCR [153, 228]. A reversible state of unresponsiveness, an ability of T cells to tune themselves to stimulus, could prove beneficial within the immune system as a means of limiting the risk of autoimmune disease through molecular mimicry [161].

Adaptation can be quintessentially summed up as *in vivo* anergy [207, 220]. There are however significant differences between anergy and adaptation, primarily the latter requires persistent stimulation, although further differences do exist at the molecular level of T cell signalling. Once the stimulus is removed the cell can return to a normal state of responsiveness [220]. Although Grossman and Paul [217] put forward the TAT model to explain the ability of T cells to maintain tolerance to self-MHC-peptide complexes by tuning their TCR signalling to match the background level of stimulus, the mechanism behind this is still largely unknown. The down-regulation of TCR signalling would in turn make a T cells harder to stimulate with subsequent challenge.

Ryan *et al* [174] initiated the approach that is expanded upon in this chapter, through the use of MBP_{Ac1-9} and APL thereof in mice expressing both the $I\text{-}A^u$ and $I\text{-}A^b$ class II molecules. Previously it had been shown that substitutions at position 4 of MBP_{Ac1-9} result in variations in binding strength with $I\text{-}A^u$; with the tyrosine substitution (4Tyr) providing a 100,000-fold higher affinity than the wild-type lysine residue [237].

In vitro these differences in affinity relate directly to antigenicity, so that the stimulus provided by the 4Tyr peptide is that much greater than that provided by the 4Lys peptide. As a result these APL can be used to provide varying levels of stimulus to transgenic Tg4 T cells (of a fixed moderate TCR affinity) and thereby allow the investigation of how the T cells react to changes in strength of stimulus.

In vivo, the immunisation of 4Tyr was seen to cause apoptosis in 70% of cells compared to just 10% with 4Lys immunisation [280]. Ryan *et al* [174] used *in vitro* analysis of these cells to show that this was activation-induced cell death as a result of Fas and FasL expression by the T cells. This was then translated into the H-$2^{u\ x\ b}$ mouse; as expected 'normal' Tg4 cells in conjunction with a 4Tyr immunisation failed to induce EAE whereas Fas-deficient Tg4 cells were able to induce EAE. However the level of disease induced was considerably less severe than that seen with a 4Lys immunisation. The fact that Fas-deficient Tg4 cells were unable to cause more severe disease opened the possibility that other mechanisms were acting to maintain tolerance. Further *in vitro* analysis confirmed *in vivo* findings that not all Tg4 cells died after challenge with 4Tyr. These remaining Tg4 cells were reduced in their proliferative potential against 4Lys and 4Tyr in a dose-dependent manner. This functional desensitisation correlated with enhanced CD5 expression [174].

CD5 has been previously shown to have a role in MOG_{35-55} induced EAE; the upregulation of CD5 correlated with T cell unresponsiveness to the MOG_{35-55} peptide [281, 282], a second study showed the resistance of CD5 knock-out mice to the induction of EAE. CD5 is known to regulate Fas expression and the absence of CD5 may have led to increased AICD within the disease relevant population of T cells. Knowing that CD5 works in conjunction with the molecules involved in TCR signalling, including CD4 and Zap70 [223, 224], it is entirely feasible that CD5 may exert its effects by dampening the level of signalling at the TCR and therefore reducing the activation potential of the relevant T cell.

The strength of signal 1 depends on the avidity of the pMHC complex to the T cell which in turn is based on the intrinsic affinity of the TCR for the pMHC complex, the density of the TCR and the density of the pMHC complexes [157]. The previous dogma predicted that (a) if the antigenic signal is too strong then relevant cells will die, (b) if the signal is too weak then the relevant cells are ignored and (c) if the signal is just right then the population of relevant cells will be expanded [161]. In both (a) and (b) the T cells cannot reach their full autoaggressive potential to mediate disease. However in (c) disease is able to develop.

This chapter investigates the effects of a strong antigenic signal on a population of T cells bearing moderate affinity TCR (Tg4 cells) and addresses whether this stimulation can lead to the functional desensitisation (adaptation) of T cells and how these changes limit the potential of auto-reactive T cells to become autoaggressive.

Investigating adaptation in the immune system is essential within T cell mediated autoimmune disease because it can help to explain the mechanisms by which the autoreactive T cell repertoire's sensitivity is kept below a 'threshold for harm' (the concentration of antigen required to make the autoreactive cells autoaggressive). This chapter develops the *in vitro* model and investigates the mechanisms behind adaptation *in vivo.* It expands on the observation that not all antigen relevant T cells die in a situation where the signal 1 is too strong in order to prevent pathogenicity and raises the prospect that self-reactive T cell repertoires can be maintained through adaptation.

5.2. MBP_{Ac1-9} superagonist APL, 4Tyr, is unable to induce disease in the C57BL/6 X B10.PLmouse despite the transfer of transgenic cells.

As shown by Ryan *et al,* the introduction of Tg4 transgenic cells into the C57BL/6 x B10.PL mouse followed by a 4Lys immunisation has the ability to induce EAE [174]. In this chapter the capacity of the superagonist 4Tyr peptide to induce disease in similar fashion was addressed. $2x10^6$ Tg4 $CD45.1^+$ cells were transferred into C57BL/6 x B10.PL mice, which were then immunised with either 100µg of 4Lys, 100µg 4Tyr or 10 µg 4Tyr (Fig. 5.1A). The mice were scored for clinical signs of EAE for 50 days (Fig 5.1B). As Figure 5.1 shows the 4Lys immunised mice presented with a standard course of EAE and those immunised with the high dose of 4Tyr showed no clinical signs of EAE until day 34, when one of the five mice developed signs of EAE. The disease course in the mouse lasted for 9 days in total reaching a maximum clinical score of 3. The fact that disease developed in this mouse suggests that initial immunisation may not have been fully effective in inducing adaptation or, to make a larger assumption, that the adaptation induced by the high dose 4Tyr immunisation was reversed after the 34 day period. The cells would then have been able to react against the 4Lys peptide present in the CNS. The ability to reverse its adaptive state is key to the theory that T cells have a tuneable activation threshold for reactivity against antigen.

Interestingly, the low dose 4Tyr immunisation group showed no significant difference in disease course for the first 25 days when compared to the 4Lys group. The 4Lys group subsequently recovered from EAE whereas the low 4Tyr group showed a prolonged disease course and the Mann-Whitney test showed a significant difference between the two disease courses between days 26 and 50 ($p<0.0001$). The fact that a low dose of 4Tyr induced disease and a high dose did not confirms the quantitative, rather than qualitative, nature of the peptide.

Mice were culled at day 50 and organs were prepared for flow cytometry. The data obtained does not support differences in Tg4 cell numbers between different immunization groups as a cause of differential disease courses. Tracking of the

CD45.1 marker showed Tg4 levels to be similar in all groups in the spleen (Fig 5.2A).

The failure of the Tg4 cells to mediate EAE with 100µg 4Tyr immunisation did not appear to be a result of regulation; the flow cytometry data (Fig 5.2B) indicated similar expression of Foxp3 in the transferred Tg4 cells between the high dose 4Tyr and 4Lys mice; hence appearing to rule out the action of regulatory T cells. There was however a significantly higher expression of Foxp3 in low dose compared to high dose 4Tyr immunisations; this may be a result of the ongoing disease in low dose 4Tyr immunised mice.

CD5 expression appeared to be dependent on antigenic signal strength (Fig 5.2C & D). Tg4 cells from both of the 4Tyr immunisations showed a higher CD5 expression than those from the 4Lys immunisation, this may be a qualitative effect of the 4Tyr APL with the differences of CD5 expression by these groups explained by the quantitative effect of dose. In the high dose 4Tyr this difference compared to 4Lys was significant (unpaired t test; $p = 0.0363$).

In vitro challenge of the splenocytes with 4Lys and 4Tyr peptides and subsequent intracellular flow cytometry staining showed that under all conditions the 4Lys immunised mice produced more IFN-γ producing cells than the mice immunised with low dose 4Tyr, which in turn produced more IFN-γ producing cells than the high dose 4Tyr mice (Fig 5.3A & C). This conflicted with the fact that the low dose mice were still ill at time of culling whereas the 4Lys mice had recovered from disease but may be because IFN-γ producing cells in the former mice are retained in the CNS as a result of ongoing disease. Interestingly this low dose 4Tyr group produced higher levels of IL-17 post *in vitro* challenge under all conditions, including with no antigen (Fig 5.3B & C), which suggests that the IL-17 may be responsible for the prolonged disease course in this group. The flow cytometry data showed no IL-10 production from Tg4 cells in any of the sets of mice (data not shown).

5.3. 4Tyr adapts T cells after a single round of stimulation *in vitro*

In order to assess the adaptation of Tg4 cells to varying levels of stimulus, over a given period of time, a series of *in vitro* experiments were performed to predict how the cells would react *in vivo* to persistent stimulation by antigen. *In vitro* experiments allowed easier monitoring of the changing phenotype and behaviour of the disease relevant cells.

Tg4 cells were stimulated (as described in Section 2.6) in 3 cycles, or rounds, over a period of twenty-one days with either 10μM of the 4Lys antigen or 1μM, 0.1μM and 0.01μM of the 4Tyr antigen (Fig 5.4). Initial experiments showed that Tg4 cells were unable to survive in short term cultures stimulated with 10μM of 4Tyr and proliferation was not sufficient to maintain cells in cultures stimulated with 1μM of 4Lys. Each round of stimulation began with the stimulation of Tg4 cells, with histocompatible APC and antigen, for 72 hours. The lymphocyte fraction was then separated out using Nycoprep density gradient and cultured, in the absence of antigen, in medium containing rat concanavalin A supernatant containing IL-2 for 4 days. The cells were then stimulated again with the same dose of peptide as before and entered the cycle again. Flow cytometric data was analysed prior to the next round of stimulation. In order to be able to compare the density of surface molecules, through mean fluorescence intensity (MFI), the data from each round had to be collated at the same time. To address this, the cultures were started sequentially, for example the 3-round cultures were started a week before the 2 round cultures. Naïve mice were culled to show data from cells that had received no peptide stimulation in culture.

Proliferation data showed that a single round of stimulation with high dose 4Tyr was sufficient to reduce the proliferation potential of Tg4 cells, compared to 4Lys stimulation, when subsequently assayed against 4Lys (Fig 5.5A). After two rounds of stimulation the reduced proliferative potential of these cells was more pronounced, with a 2 log shift in the concentration of peptide required to initiate proliferation (Fig

5.5B). A third round of stimulation showed only a single log shift – similar to that seen after one round of stimulation (Fig 5.5C).

When assayed with the 4Tyr peptide all the short term cultures appeared to react in a similar fashion after the first round of stimulation (Fig 5.5D). After the second round and third rounds, however, 4Lys cultures were seen to respond at very low concentrations of the peptide, starting at 0.001μM (Fig 5.5E & F) As the peptide concentration was increased the proliferative potential of these cells was reduced compared to the lower peptide concentrations. The 4Tyr cultures did not appear to reduce as dramatically in proliferation potential to 4Tyr challenge in these subsequent rounds suggesting that these cells had adapted to the stronger stimulus.

5.4. 4Tyr induces phenotypic changes in cultured Tg4 cells

Cell surface markers were evaluated using flow cytometry to identify those cells potentially involved in or indicative of an adaptive response. Analysis showed consistency with proliferation data and showed decreases in TCR levels after a single round of stimulation in 4Tyr stimulated cell lines whereas the 4Lys line showed an increase in TCR when compared to the naïve control cells shown on the x-axis (Fig 5.6B). The decreases in TCR in the 4Tyr lines were dose dependent; the high dose 4Tyr cell lines showed a 46% reduction of TCR compared to naïve Tg4 cells (i.e. 0 rounds of stimulation cells shown in Fig 5.6) and a 54% reduction when compared to those stimulated with 4Lys. For each 10-fold reduction in the 4Tyr dose we observed a one-third increase in TCR levels.

As Figure 5.4 shows, flow cytometric profiles were taken at day 6 during each cycle of stimulation. This enabled the elimination of non-relevant cells and allowed the examination of adapted T cells in a resting state. After a single round of stimulation, levels of CD5 were increased in all stimulated cell cultures when compared to the levels on naïve Tg4 cells (Fig 5.7A & B). There was an immediate difference

between the 4Tyr and the 4Lys short term cultures which became more pronounced after the second round of stimulation. The high dose 4Tyr cultures had a CD5 MFI 28% greater than the 4Lys cultures after a single round of stimulation; this rose to 67% after two rounds however analysis after a third and final round of stimulation showed CD5 levels in all short term cultures returning to similar levels to the naïve cell controls.

The changes in the expression of CD5 appeared to correlate to the strength of the stimulus; 4Lys short term cultures showed the least increase of any cell line and in the other lines as 4Tyr dose increased so did the CD5 levels. Levels of CD25 and CD44 increased to similar amounts in all cultures, which suggested no difference in activation status of the cells (data not shown).

The changes observed in these short term cultures presumably contribute to the reduced sensitivity of the T cell following superagonist stimulation; changes in cells stimulated with 4Lys peptide can be explained by the fact that the concentration of 4Lys peptide introduced to these cultures is far greater than the normal levels that would be seen *in vivo*. The data suggest that the initial suppression in proliferation capacity of 4Tyr cultured cells is mediated by a loss in TCR. More dramatic suppression correlates with the later increased level of CD5, with the exact role of CD5 being undetermined. The fact that CD5 levels drop after a third round of stimulation may point to a role for the molecule only in the early onset of adaptation, mediating the process but not taking an active part in maintaining the adaptive state of tolerance.

5.5. Single round 4Tyr stimulated Tg4 cells are pathogenic *in vivo*

Previous data from the Anderton group show that a single round of stimulation of Tg4 cells with 4Lys *in vitro* confers a pathogenicity to the cells which is manifested

as disease when cells are transferred into the B10.PL mouse (unpublished observations).

This protocol was used to investigate how adapted cells acted *in vivo* and to ascertain whether *in vitro* adapted cells could migrate to the CNS and could thereby cause disease (Fig 5.8A). The question of how long a state of unresponsiveness could last was also addressed by this method. The same protocol for stimulating cells was used as described previously in Chapter 3.5.

Results showed that cells stimulated with both high and low dose 4Tyr had a significantly greater pathogenic potential than the 4Lys stimulated cells (p=0.0152 and p=0.0209 respectively; Mann-Whitney test). As Figure 5.8B indicates, although disease onset was similar between the groups, it was the point of recovery that was noticeably different; in the 4Lys group only one mouse was still ill after day 17 with a clinical score of just 1. All the mice in both superagonist stimulated groups presented signs of EAE till the endpoint (day 25).

Tg4 cells could be tracked with the CD45.1 marker and flow cytometric analysis of the spleen at the peak of disease (day 14; Fig 5.9A) showed a 75.9% increase in the level of Tg4 cells present in the 4Lys group compared to the high dose 4Tyr group; this was shown to be on the cusp of significance using an unpaired t test, where p = 0.0479. There was also a significant 3-fold increase in the 4Lys group compared to the low dose (p = 0.0048). Examination of the CNS showed in all groups that there was an enrichment of Tg4 cells in the spleen compared to the CNS however there was no significant difference between the groups in terms of the levels of Tg4 cells present.

CD5 levels on Tg4 cells in the spleen were similar in all groups, with similarities observed in Tg4 cells recovered from the CNS also (Fig 5.9C). Tg4 cells in the CNS had a higher expression of CD5 compared to those found in the spleen; it may be that activated cells migrate to the CNS despite levels of adaptation. Lower percentages of

4Tyr stimulated Tg4 cells may be found in the CNS as the peptide is not expressed there.

5.6. Repeated *in vitro* stimulation is required to prevent pathology *in vivo*

Tg4 cells were stimulated for two rounds *in vitro* with 10µM 4Lys, 1µM 4Tyr or 0.1µM 4Tyr. Then, $2x10^6$ cells were transferred into C57BL/6 x B10.PL mice and two doses of PTx were administered (Fig. 5.10A).

Figure 5.10B shows data collated from two separate experiments. Cells stimulated with 0.1µM 4Tyr were able to induce EAE of a similar severity to these stimulated with 10µM 4Lys (no significant difference - Mann-Whitney: $p = 0.6344$). A dose of 1µM 4Tyr was also able to mediate disease but to a significantly lower level than both the 4Lys and the low dose 4Tyr stimulated cells (Mann-Whitney: $p = 0.0002$ and $p = 0.0009$ respectively). This suggested a limited pathogenic potential of these cells exposed twice to 1µM 4Tyr.

Tracking of the $CD45.1^+$ Tg4 cells via flow cytometry showed that at the peak of disease, day 14, the percentage of Tg4 cells in the spleen was 50% lower in the 1µM 4Tyr group compared to the 4Lys group; this observation was matched in the CNS (Fig. 5.11A). Although not shown to be significantly different (unpaired t test analysis), it is still difficult to conclude that the lower severity of disease was a result of adaptation and it may simply have been a feature of the reduced levels of cells present to mediate disease. The reduced expansion of the 4Tyr stimulated Tg4 cells may be a result of the lack of *in vivo* stimulation as exposure to the 4Lys peptide present in the CNS may not provide strong enough stimulation to maintain the transferred cells, if their sensitivity had been lowered *in vitro*.

Further analysis showed no significant differences in Foxp3 expression of host or Tg4 cells in either group within the spleen. Both the host and transferred cells within the CNS of the 4Lys group had more than double the mean Foxp3 positive cells than

the 4Tyr group did (Fig. 5.11B). These differences would appear to rule out increased regulation within the CNS as a cause of reduced severity of disease in the 4Tyr group.

There was no significant difference in CD5 levels (p=0.7491; unpaired t test) that could help to explain the lower severity of disease in the 4Tyr groups, similar to observations from *in vitro* experiments (Fig. 5.12A). TCR levels did however appear significantly lower in this group compared to the 4Lys (p=0.0410; unpaired t test) and it is feasible that this mechanism acts to functionally desensitise the disease relevant cells, thereby ameliorating disease (Fig. 5.12B).

5.7. Primary immunisation with 4Tyr ameliorates EAE induced with 4Lys peptide

In order to examine whether a primary superagonist challenge would have a modulatory effect on a cell population subsequently challenged with the wild-type peptide, a double-immunisation protocol was used [269] (Fig 5.13A). Either 100µg 4Lys or 100µg 4Tyr was introduced sub-cutaneously with CpG and IFA into C57BL/6 x B10.PL mice. Twenty-eight days later the mice were re-immunised with 100µg 4Lys with CFA alongside to doses of PTx. This model was also used to investigate how long the effects of adaptive tolerance could be observed. Preliminary work had shown increases in CD5 expression after the primary immunisation with CpG and IFA hence these subsequent experiments assumed adaptive tolerance would be induced in these circumstances (data not shown). It should be noted that no Tg4 cells were used in these initial experiments.

Disease onset in the 4Lys immunised group was observed a mean of two days earlier than in the 4Tyr group and the mice in the latter group appeared to expand a cohort of T cells that were reduced in their efficacy of inducing EAE (Fig 5.13B). Only 50% of these mice showed clinical signs of EAE, with a mean maximum disease score of

one. Disease lasted a mean of 3 days in this group whereas the 4Lys mice still showed clinical signs 20 days after onset of disease (when they were culled having shown a mean 9.25 days of disease). The 4Lys group showed a mean maximum disease score of 2.5, with 75% of the mice showing signs of EAE. Mann-Whitney analysis showed the difference in disease burden to be highly significant with $p<0.0001$.

Splenocytes were taken from the mice at day 27 and *in vitro* proliferation assays were performed using the 4Lys peptide. Cells from mice, immunised with 4Tyr where either no or low grade disease was observed, required there to be a 10-fold higher concentration of 4Lys to respond compared to those immunised with 4Lys (Fig 5.14A). The single mouse in which severe EAE was seen (maximum clinical score of 3) in the 4Tyr group showed a similar *in vitro* response to mice within the 4Lys group (Fig 5.14B).

A higher dose of 4Lys challenge was also required to induce production of IL-2 by these cells (Fig 5.14C). There appeared to be no defect in terms of the IFN-γ production however, with both sets of cells produced similar levels of the cytokine upon challenge (Fig 5.14D).

These data support the idea that the initial exposure to 4Tyr would be able to modulate subsequent disease course by adapting the 4Lys relevant repertoire of T cells by increasing their thresholds of proliferation and hence leaving them less susceptible to 4Lys challenge. There was no change in the sensitivity to IFN-γ production which may suggest that thresholds of activation were unaffected. In the 4Tyr group where disease was seen in one mouse it may be feasible that either the initial dose of 4Tyr was not sufficient to adapt the 4Lys relevant T cell repertoire or that the adaptive state had been reversed in the 28 day period between immunisations.

5.8. 4Tyr Immunisation following a Primary 4Lys Immunisation fails to induce EAE

To address how a diverse repertoire of T cells expanded by the primary 4Lys immunisation would react against a secondary stimulation with the superagonist 4Tyr peptide, the previous experimental protocol was reversed. Mice were immunised initially with 4Lys, in CpG and IFA. This was followed by either 100µg 4Lys, 100µg 4Tyr or 10µg 4Tyr, in CFA to induce EAE (Fig. 5.15A) with the disease course monitored for 23 days (Fig. 5.15B).

No mice in the high dose 4Tyr immunisation group showed clinical signs of EAE. Disease onset in the 4Lys immunised group was seen two days later than in the 10µg 4Tyr group. Only 60% of the mice in both these groups showed clinical signs of EAE, with the 10µg 4Tyr group having a mean maximum disease score of 2.4 compared to a score of 1.8 in the 4Lys immunised group. Disease lasted an average of 6.2 days in the 10µg 4Tyr group whereas the 4Lys mice showed clinical signs for 7.4 however disease between these two groups was shown not to be significant (Mann-Whitney analysis; $p = 0.0696$).

Splenocytes were taken from the mice at day 23 and *in vitro* proliferation assays were performed against the 4Lys peptide (Fig. 5.16). Cells from the 100µg 4Tyr group showed limited proliferation against an *in vitro* challenge of 4Lys whereas 10µg 4Tyr immunised splenocytes showed a similar proliferative potential to those from 4Lys immunised mice.

5.9. Tracking Tg4 cells within the C57BL/6 X B10.PL adaptation model

In order to track and observe behavioural and phenotypic responses in the relevant T cell repertoire during the process of adaptation $2x10^6$ Tg4 cells were transferred into the C57BL/6 x B10.PL double immunisation model (Fig 5.17A). As in previous

work (Fig 5.13), suppression of disease was seen in the groups that had received the 4Tyr compared to the 4Lys as primary immunisation (Fig 5.17B & C). This suppression appeared to be linked to dosage; in groups where a higher dose of 4Tyr (200µg) was given almost total suppression of disease was seen (only 11.1% of the group - 2 out of 18 - showed clinical signs of EAE as opposed to 100% in the 4Lys group) (Fig 5.17B). When the peptide dose was halved, 66% of the 4Tyr group showed clinical signs of disease compared to 78% in the 4Lys groups (Fig 5.17C). The dose of 4Lys did not appear to make a difference to the peak of the mean clinical score between experiments however the higher dose did appear to prolong the disease course.

Analysis of the transferred cells in the lymph nodes at seven days post primary immunisation showed similar levels of Tg4 cells (Fig. 5.18A). Phenotypic analysis of the Tg4 cells from the 4Tyr immunised groups showed that, although there was no difference in TCRβ expression (Fig. 5.18B), there was a significantly higher expression of CD5 than in the 4Lys immunised groups (Fig 5.18C; $p = 0.0046$). The increased CD5 levels in the 4Tyr group indicate that these Tg4 cells had adapted to the superagonist stimulus and this reduced sensitivity would explain a decreased pathogenic potential *in vivo* and the lower severity of disease. There was no significant difference in either CD44 (Fig 5.18D) or CD25 (Fig 5.18E) expression ($p=0.4529$ and $p=0.5704$ respectively; unpaired t test). Similar CD25 levels indicate no enhanced role for regulatory T cells with the caveat that Foxp3 analysis was not performed as part of these experiments as it was not deemed relevant at that time.

When splenocytes from mice are examined at peak of disease after the secondary immunisation (day 14) the frequency of Tg4 cells is significantly higher in the 4Lys (Fig. 5.19A); with around double the frequency of cells present compared to the 4Tyr group ($p=0.0054$; unpaired t test). This significant difference is also observed in the levels of total $CD4^+$ cells within the CNS (Fig 5.19B; $p=0.0007$; unpaired t test). However, Tg4 cells were not identifiable in the CNS at this time-point.

There was no significant difference in expression of TCR (Fig. 5.19C) or CD5 (Fig. 5.12D) between the two groups (p=0.5230 and p=0.6135 respectively; unpaired t test) which could have helped to confirm the adapted state of the Tg4 cells present in the 4Tyr group.

Data in this chapter show that in 4Tyr immunised mice, Tg4 cells do not track as effectively to the CNS as in 4Lys immunised mice. This may be reflective of the decreased numbers in the periphery but also because the 4Tyr is not presented within the CNS. CD44 levels in cells from the 4Tyr mice were significantly higher (Fig 5.19E; p=0.0272) and this can be explained by the superagonist properties of the 4Tyr peptide. This would indicate that it was not a failure of the 4Tyr antigen to activate the Tg4 cells that led to a reduction in disease severity. However the reduced numbers of cells in the spleen and CNS may thus be due to activation induced cell death caused by the potency of the 4Tyr peptide.

Proliferation data from splenocytes (taken either at the peak of disease and after clinical signs have ceased) show that cells from 4Tyr immunised mice were defective in their ability to respond to the 4Lys peptide *in vitro* compared to cells from mice immunised with 4Lys (Fig 5.20). Bearing in mind that the initial *in vivo* immunisation was with 100µg of 4Tyr it would seem reasonable to extrapolate that the secondary *in vivo* stimulation of these cells with 100µg of 4Lys may not be enough to efficiently drive their expansion leading to less severe EAE, as seen in Figures 5.17B and C.

5.10. Discussion

It has been proposed that the activation of auto-reactive T cells can be explained by two mechanisms; one of these, epitope spreading, was discussed in chapter 4; the second, molecular mimicry, is addressed by the series of experiments presented within this chapter. The data contained within this chapter indicate a role for adaptation as one the mechanisms of tolerance at play in ameliorating disease and maintaining a repertoire of disease relevant T cells.

In line with previous work [174], the data within this chapter show that stimulation with a superagonist peptide (4Tyr) through immunisation is unable to induce disease when administered at the same dose as the wild-type (4Lys). However, if the dose of 4Tyr was reduced 10-fold, compared to 4Lys, then the disease course induced was similar to that observed with the 4Lys. The fact that a reduction in the load of 4Tyr causes EAE would appear to uphold the tuneable activation threshold model; the cells will have received a lesser degree of stimulation with the lower dose and are therefore able to react against the 4Lys self-antigen present in the CNS. Data show that high doses of 4Tyr did not induce cell death of all the transferred Tg4 cells even by day 50 (Fig. 5.2A), hence the autoreactive repertoire of cells was maintained. The flow cytometry data indicate a role for CD5 and potentially adaptation in facilitating this, again agreeing with previous observations [174].

An interesting observation here is the increased level of effector cytokine activity from low dose 4Tyr immunised mice; there was an increase in the percentage of Tg4 $CD4^+$ cells recovered from these mice that produced either IFN-γ or IL-17, compared to those recovered from high dose 4Tyr or 4Lys immunised mice. This was seen even in the absence of *in vitro* peptide challenge and may explain why the low dose immunisation is so effective at potentiating disease for longer than the 4Lys immunisation.

The work with Tg4 cells *in vitro* (Fig. 5.4) allowed the examination of the changing behaviour and phenotype of the cells in a far more simplified environment, compared to the *in vivo* situation, and free of non-relevant $CD4^+$ cells. Again the data show a limitation in the pathogenic potential of Tg4 cells stimulated by high dose 4Tyr, whereas stimulation with low doses of 4Tyr produced cells with a similar pathogenic potential to 4Lys. A reduction in TCR expression and an increase in CD5 expression may both combine to allow T cells to reduce their functional sensitivity for 4Lys and thereby avoid AICD. Both of these observations are supported by previous studies, although what was not observed here was a reduction of CD4 expression, as seen elsewhere [283, 284]. Further work is also required to examine possible reductions in Ca^{2+} signalling and MAPK activation and the potential effects on the production of cytokines.

The fact that, during the second and third rounds of stimulation, high dose 4Tyr stimulated cells were seen to respond to the 4Lys at 1μM supported the theory that it is selective pressure which pushes $CD4^+$ cells to respond to antigen at this pre-determined threshold; in this scenario the pressure having induced adaptation [159, 260].

When these 4Tyr cultured cells were examined *in vivo*, using the passive transfer model (Figs. 5.8 & 5.9), at least two rounds of *in vitro* stimulation were required before their pathogenic potential was limited, compared to 4Lys stimulated Tg4 cells. This would support the idea that sustained stimulation is required for adaptation to occur. The pathogenic activity of cells exposed to a single round of 4Tyr stimulation may be explained by three factors; the cells found in the CNS may not have been fully adapted *in vitro*, adaptation may have been reversed when the superagonist stimulation was no longer present or the fact that cells were transferred immediately after the polarising conditions of the primary stimulation and prior to a rest period.

The observation that 4Tyr mediated disease in the two round stimulation experiments is reduced in severity is clouded by the fact that the numbers of Tg4 cells recovered in the 4Tyr groups are halved compared to the 4Lys groups. This may be the

explanation for the difference in disease course seen mediated by the persistently stimulated cells. The poor viability of the Tg4 cells in the 4Tyr high dose groups may be in turn be explained by the weaker stimulation they should receive *in vivo* from wild-type 4Lys presented in the CNS resulting in cell death.

A dual-immunisation protocol enables the cross-reactive activation of auto-reactive T cells using the APL system. Unlike in previous experiments with the C57BL/6 x B10.PL mice, this chapter shows the manifestation of disease without the use of transgenic cells. There is pre-existing data that suggests that the repertoire of T cells expanded by the initial immunisation, the antigen-experienced cohort, are more liable to cause disease than the naïve cohort would have been [285]. This is largely due to less dependency for co-stimulation by B7 molecules and CD40.

When 4Tyr was administered as a primary immunisation to be followed by 4Lys, the experiments attempted to mimic a scenario where T cells are activated by a strong stimulus from a foreign epitope followed by a weaker stimulus from a self epitope. The experiments show disease course was significantly decreased and splenocytes from these mice show a limited proliferative potential when challenged *in vitro* compared with cells from 4Lys primed mice. This supports the theory that antigen experienced cells tune themselves to a particular stimulus and that they require this level of stimulus to become activated again, at least for a certain period of time after the initial stimulus. The data here confirm previous observations in a $PLP_{139-151}$ APL Y144/L144 model (where residue 144 is replaced by either tyrosine or leucine [286]) and show that IL-2 production is impaired in splenocytes from 4Tyr immunised mice compared to 4Lys groups whereas the level of IFN-γ produced is similar in both. There may be a mechanistic reason behind this: The factors controlling adaptation may only exert effects on the pathways which control the transcription of IL-2 and not those that control IFN-γ. This is however in contrast to data from Singh *et al* which predict that both IL-2 and IFN-γ production should be impaired in adapted cells [219].

Accepting the caveat that they refer to this state of non-responsiveness as anergy rather than adaptation, Munder *et al* [286] explored the avenue of T cell signalling which was not investigated here. Activation of relevant T cells with Y144/L144 led to an unresponsiveness characterised by a dramatic reduction in tyrosine phosphorylation of TCR chains as well as a diminished Zap70 phosphorylation and Ca^{2+} signalling. These observations combine well with the theory that CD5 forms a complex with CD4 and Zap70 in adapted cells and that reduced levels of CD5 correlate with increased levels of Ca^{2+} signalling and Zap70 phosphorylation [223, 224]. This would appear to endorse the use of CD5 as one, of potentially multiple, marker of adaptation in these data.

In the reverse scenario, where 4Tyr immunisation followed a 4Lys primary immunisation, the experiment mimics a scenario where an antigen experienced cohort of T cells is challenged subsequently with a much stronger stimulus. In these experiments no disease is observed after the high dose 4Tyr secondary immunisation. The low dose 4Tyr secondary immunisation group however showed similar disease to the 4Lys secondary immunisation group. Again, in terms of the level of stimulation provided to activate the T cells, the low dose 4Tyr provided a similar stimulus to the 4Lys. This highlights the potential for non-self peptides to promote disease by acting on a cohort of auto-aggressive cells expressing cross-reactive TCR.

When transgenic cells were placed into this model, to enable clear tracking of the antigen-relevant cells, differences were evident mainly after the primary immunisation in cells taken from the lymph nodes. Once again it was the expression of the CD5 marker that was elevated on the 4Tyr stimulated cells with no other markers tested showing differences. Severity of disease was again reduced in the 4Tyr immunised mice and although Tg4 numbers after disease may not reflect Tg4 number during disease the differences in pathogenic potential of the cells may have been largely numerical. There were less than half the numbers of Tg4 cells in the spleen of 4Tyr immunised mice compared to the 4Lys immunised mice after disease. This may be a result of less stimulation for these cells throughout the 52 day experimental timecourse, meaning that many are unable to survive.

Chapter 6:

General Discussion

6. General Discussion

The experiments in this thesis use TCR transgenic models to explore two paradigms in autoimmune disease, namely epitope spreading and adaptive tolerance. Epitope spreading is the diversification of the immune system to an epitope distinct from the initiating epitope. Adaptive tolerance is based on the tuneable activation threshold model which suggests that a T cell repertoire with an autoreactive nature can tune its threshold for harm to prevent progression to an autoaggressive nature.

6.1. The role of epitope spreading and the potential for bystander suppression in autoimmunity

Epitope spreading is the concept used to explain the relapsing-remitting form of MS and EAE; predicting that the immune system's focus against one epitope may cause damage in the target organ resulting in the release of cryptic self epitopes to which relevant effector T cells can react, thereby mediating a diversification of the immune response. The immune response to any of the epitopes involved may fade as disease progresses and this may make the identification of such epitopes more difficult.

The data in Chapter 4 shows that the introduction of Tg4 cells into the B10.PLxSJL mouse has a pronounced effect on disease. Only in the presence of the Tg4 cells do the mice relapse into a secondary phase of disease and yet none of the *ex vivo* data show cell response against the 4Lys peptide, nor does flow cytometric data show the presence of Tg4 cells in the CNS of sick mice. It is possible that the response of the immune system to the MBP_{Ac1-9} epitope may be imperceptible to our detection techniques, or that the response may fade rapidly and spread to other epitopes. Data in Chapter 4 showing pathogenicity of the MBP_{Ac1-9} epitope in B10.PL x SJL mice are consistent with previous findings showing that the epitope causes RR-EAE [265]. This suggests that relevant MBP_{Ac1-9} T cells must have the capacity to travel into the CNS of these mice and that perhaps the chosen timing of the analysis looking for the cells in the data shown here was unfortunate. Further investigation of

this mouse, with $PLP_{139-151}$ immunisation and Tg4 adoptive transfer is required, prior to relapse, at the peak of each phase of disease and during recovery periods to address whether reactivity can be detected from cells in the lymph nodes, spleen and the CNS; cell infiltration into the CNS must also be confirmed.

It therefore stands to reason that the epitope hierarchy in spreading within this model must be investigated. If the immune response to MBP_{Ac1-9} does fade rapidly then the epitopes to which the immune response diverts to must be identified. Previous data in the SJL mouse show that $PLP_{139-151}$ immunisation resulted in RR-EAE in which the hierarchy spread intramolecularly to a primary relapse against $PLP_{178-191}$ and then intermolecularly, for a second relapse against the MBP_{84-104} epitope [267]. Intramolecular spreading in the B10.PL x SJL mouse was seen by Lehmann *et al* [265]. Splenocytes from these mice were shown to have recall responses against MBP epitopes 35-47, 81-100 and 121-140. An effective strategy to address the hierarchy question would involve *ex vivo* proliferation assays against all the epitopes mentioned above at several time points during disease, prior to, during and in recovery of each phase of disease.

Bystander suppression occurs when regulatory T cells are generated relevant to one epitope but have the potential to regulate or suppress disease mediated by cells relevant to a different non-structurally related epitope that is present within the same organ or tissue. Hence the diversification of the immune response seen in epitope spreading may be advantageous if regulatory T cells relevant to revealed epitopes can be used to down regulate disease directed against another epitope. The experiments shown in Chapter 4 use the 4Tyr APL of MBP_{Ac1-9} with PBS within the Tg4 cell / B10.PLxSJL model prior to the induction of EAE with $PLP_{139-151}$ and CFA. The data show the absence of a relapse between days 20 and 45 in the 4Tyr treated mice. Further analysis of tissues shows that not only are Tg4 cells enriched within the CNS samples but that 45% of these cells expressed the regulatory T cell marker, Foxp3.

The data shown, however, do not address the question of whether the 4Tyr induced regulatory Tg4 cells are mediating bystander suppression in this model. No data assessing cytokine production of these cells were collated and therefore it cannot be discerned whether Tg4 Foxp3$^+$ cells have a direct role, through the production of IL-10 or TGF-β, in the down-regulation of pathogenicity of disease relevant effector T cells. Again, a major caveat here is the absence of evidence showing T cell reactivity against 4Lys in *ex vivo* assessments.

One serious critique of these experiments may be the difference in time points of the introduction of Tg4 cells (Figure 4.6A). Control mice not receiving 4Tyr were given Tg4 cell transfer 6 days after mice that did receive 4Tyr treatment. This was justified due to concerns about the viability of Tg4 cells in the B10.PL x SJL mouse in the absence of TCR stimulation.

Experiments must be planned to address whether 4Tyr exposed / induced regulatory Tg4 cells are able to effectively suppress disease induced with epitopes other than $PLP_{139\text{-}151}$. This can be simply addressed by immunisation with whole myelin.

The role of the APC must also be examined. Recent data suggest that three populations of DCs are recruited into the CNS from the periphery during acute phases of disease in relapsing-remitting EAE; mDCs, pDCs and CD8α DCs [287]. It may be possible that one subset of DC is more efficient as APC for an effective T cell response to MBP [119] and another subset is more efficient for $PLP_{139\text{-}151}$. In that scenario, Tg4 cells migrating to the CNS may therefore be left at a disadvantage. Firstly the immune response in the $PLP_{139\text{-}151}$ immunised model would encourage the active recruitment of PLP relevant DC to the CNS; secondly the cytokine milieu created by that subset of DC may negatively affect the antigen presenting capacity of another group of APC needed to mediate MBP relevant disease. It stands to reason that if certain APC are essential in epitope spreading then others may be crucial in the induction of bystander suppression. This in turn brings us to the role of cytokines in RR-EAE; what is the ideal cytokine milieu for epitope spreading to occur? Cytokine production from CNS cell infiltrate also needs to be investigated.

Bystander suppression may be the answer to the how T cells can be tolerised against known and unknown epitopes in autoimmune disease. If regulatory T cells can be produced against a key disease relevant epitope then they may afford the control of disease that is directed against any epitope.

6.2. Investigating T cell tuning with a molecular mimicry model of disease

The experiments presented in this thesis used APL to assess how a state of tolerance may be both maintained and potentially broken by molecular mimicry. The APL / EAE model does not allow the evaluation of microbially-derived epitopes. However it does help to elucidate the mechanisms behind the molecular mimicry based initiation of autoimmune disease as well as disease events after such an initiation. This, in turn, will help to design strategies for the treatment and prevention of autoimmune diseases.

The suggestion that antigenic determinants of micro organisms, specifically parasites, resembled antigenic determinants of hosts was first made in the 1960s by Damian *et al* [288]. This molecular mimicry concept was initially used to explain how parasites evaded host immune responses to survive and was later modified to suggest immune responses against a pathogenic epitope could at a later time point develop into autoaggressive responses through recognition of host epitopes bearing structural or sequence homology to the pathogenic epitope [228]; with the initiating event and autoimmune onset separated temporally and spatially.

Although the $MBP_{Ac1\text{-}9}$ APL model is limited by differences in disease induced by the various APL, it can be used to mimic a situation where a relevant T cell repertoire has been challenged by a microbial epitope giving a strong stimulus (4Tyr) to relevant T cells. These T cells can, at a later time point, be challenged by weaker stimulation with a self epitope (4Lys) bearing structural homology to the initiating

epitope. This could also be reversed to model a scenario where an antigen experienced repertoire of T cells is created by a foreign epitope (4Lys) giving a weak signal to the TCR and then stimulated by a self epitope giving a much stronger stimulus (4Tyr).

Wucherpfennig and Strominger searched for structural homology between viral and bacterial peptides by analysing epitopes in which TCR-binding amino acid residues were similar to those from MBP. From a panel of 129 epitopes identified and tested on seven MBP-relevant T cell clones, from MS patients, seven viral epitopes and one bacterial epitope were able to activate three of the clones. Interestingly, only one epitope could have been identified through sequence alignment [232]. The clearest example of molecular mimicry in a model of MS is shown in the series of work using Theiler's virus genetically modified to express epitopes activating PLP relevant T cells [52, 55, 277] and has been previously discussed (Section 1.3). EAE investigations have found that homology between microbe and mimic do not have to be extensive, with a polyalanine peptide sharing only 4 residues with MBP_{Ac1-9} able to induce EAE in PLSJL/J F_1 mice [289]. Semliki Forest virus and MOG epitopes have also been shown to show homology and induce MS-like disease through this mimicry [290]. Antiserum against $MBP_{110-124}$ shows reactivity against *Pseudomonas* peptides as well as against *Acineobacter* peptides, antiserum against MOG_{43-57} also showed reactivity against *Acineobacter* peptides [291].

The work shown here examines molecular mimicry in the setting of adaptive tolerance using a simple model based on immunisation with peptides and APL rather than infection. The investigation is built on previous work which highlights the effect of superagonists [174, 261]. Adaptive tolerance may be an appealing option for the immune system compared to cell death because the reversibility of the state affords the option to maintain a diverse T cell repertoire; it is a repertoire that is kept below a threshold for harm. Reversibility allows the maintenance of disease relevant T cells which may bear cross-reactive TCR needed at a later time-point for responses to microbial epitopes. Paradoxically, reversibility may be the Achilles' heel of adaptive tolerance; if a microbial epitope is able to expand a large population of autoreactive

T cells which are adapted then, after a period of time when the cells regress from the adapted state, they could become autoaggressive and react against self-peptides which mimic the initiating peptide. This is dependent, however, on a later insult to the target organ. In the case of MS and EAE, a requirement of perturbation of the blood brain barrier and insult to the CNS.

The data shown here indicate the potential for APL to prevent autoimmune disease by inducing adaptive tolerance amongst autoreactive cells and thereby preventing them from becoming autoaggressive. There are several caveats to the data shown here, primarily that the other two key mechanisms of tolerance are not fully ruled out. The effects of the Fas / FasL mediated AICD and the capacities of regulatory T cells have not been eliminated from the analysis. The possibility that the culture conditions merely help to select a distinct TCR bearing repertoire from the Tg4 $CD4^+$ population used is also yet to be eliminated. Several attempts were made to address these issues; experimental procedures outlined in Figure 5.4 were attempted in a more simplistic form, using only 1mM 4Lys and 1mM 4Tyr *in vitro* stimulations, to compare changes in 'normal' Tg4, Tg4 *lpr* (mutant Fas deficient mice) and Tg4 $RAG^{-/-}$ mice.

Tg4 *lpr* mice allow the exclusion of AICD due to their Fas deficiency and would allow determination of whether adaptation alone is sufficient to limit the pathogenic potential of disease relevant cells. These mice could also be used to investigate whether cells with low expression of CD5 are merely deleted from the repertoire leaving only cells with high CD5 expression or if in fact de novo increases in CD5 expression occurs on adapted cells in line with the level of stimulus received in the immediate environment. If CD5 expression increases upon 4Tyr stimulation in Tg4 *lpr* cells then it would suggest that adaptation is accompanied by an increase in CD5 expression. If only a small number of Tg4 *lpr* cells express CD5 then it could be suggested that the elevation of CD5 shown in the data within this thesis is a result of the loss of Tg4 cells with low expression of CD5.

Theoretically, Tg4 cells bear a moderate affinity TCR for 4Lys. However, unless these mice are crossed onto the $RAG^{-/-}$ background, the possibility of different repertoires of Tg4 cells bearing different affinity TCR for 4Lys still exists. RAG sufficient Tg4 cells may have the ability to express alternative α or β chains. The Tg4 $RAG^{-/-}$ mice allow the confirmation that 4Tyr limits the pathogenicity of the Tg4 cell repertoire towards 4Lys through adaptation rather than selection of low affinity TCR bearing Tg4 cells which bind poorly to 4Lys-MHC complexes. It was not possible, however, to keep either Tg4 *lpr* or Tg4 $RAG^{-/-}$ cells alive within cultures, regardless of stimulating peptide. This may be explained by the poor conditions of the animal unit in which these mice were kept. Cells from Tg4 mice which were kept in a separate unit did not present with the same problems.

It appears that only Schwartz's original demonstration of adaptation [219], using $RAG^{-/-}$ transgenic peptide relevant cell transfers into $CD3\varepsilon^{-/-}$ mice have been able to rule out the effect of regulation and the selection of distinct repertoires; as this is the only *in vivo* adaptation model that uses only antigen relevant T cells of the $RAG^{-/-}$ background and can therefore preclude the host T cells from the analysis.

This system also shows an increase in CD5 expression in $CD4^+$ T cells following the induction of tolerance; these studies associate CD5 with the SHP-1 signalling molecule. CD5 expression has also been used as a part of the analysis of an adaptive state within the experiments shown here. The majority of the data here show that T cell unresponsiveness correlates with an increase in CD5 expression. However the role of this glycoprotein is not fully clarified. Previous data has highlighted a role for CD5 within the EAE model. Elevated CD5 expression is associated with T cell unresponsiveness when MOG_{35-55} is presented by steady-state DC [281] and apoptosis of disease-relevant cells is increased in the absence of inhibitory signals from CD5 in knockout mice to suppress EAE [282]. These and other data show a negative regulatory role for CD5 in T cell activation [292] which is mediated by the cytoplasmic region [293] independently of extracellular engagement [294]. CD5 has also been implicated in studies of adaptive tolerance with $CD8^+$ cells [295].

CD5 is upregulated alongside CD6 and CD2 on activated T cells. CD5 shares similarities with the CD6 molecule also, for example both contain several tyrosine residues in their cytoplasmic regions. An interesting avenue of exploration is the role of CD6 alongside CD5 as CD6 interacts with the CD166 ligand to reduce IL-2 production and has also been shown to interact with the SLP-76 SH2 domain. The elevation of CD6 on activated T cells results in unresponsiveness of T cells in a similar fashion to CD5 [296]. Even if CD5 proves to be important in our studies of MBP_{Ac1-9} APL, it may eventuate that CD5 may not be required for adaptation in other models of disease or even within other models of EAE.

Previously the use of APL in models of EAE has resulted in the deviation of Th_1 responses to Th_2 responses. The L144/R147 analogue of $PLP_{139-151}$ was used to antagonise TCR and thereby prevent the activation of Th_1 cells by polarising cells to a Th_2 subtype. Primary immunisation with this analogue prevented EAE induced with a secondary immunisation with PLP, MOG and MBP [297]. APL of MBP_{87-99} have been also shown to ameliorate EAE by deviating a Th_1 response to Th_2 in mice and rats [267]. APL for the same region of MBP have been shown to prevent the production of IL-2, IFN-γ, IL-4, IL-10 and yet induce the production of TGF-β1. Investigations as part of the work shown in this thesis conflicted with previous work by Pearson *et al* which suggested that 4Tyr stimulation of MBP-specific TCR transgenic resulted in Th_2 deviation of the immune response characterised by production of IL-4 [298]. No deviation towards a Th_2 phenotype could be detected in the course of this work; Tg4 cells were stimulated with superagonist 4Tyr *in vitro* and IL-4 production could not be detected by either supernatant ELISA or flow cytometry (intracellular cytokine staining).

There are still some outstanding questions regarding the exact role and contributions of adaptive tolerance in limiting the autoaggressive potential of autoreactive T cells. What are the contributing mechanisms for adaptation or is there a single mechanism? How long does the state of adapted tolerance last and what factors cause variations in this time frame?

The APL based therapies shown in this thesis are within relatively simple systems. The translation of these therapies into human disease may be prohibitively complex and require extreme caution. APL studies are advantageous, however, in determining which disease relevant cells are more prone to tolerisation and in addressing the role of modified self antigens in autoimmune pathogenesis.

Chapter 7:

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